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TITLE: Low Level Exposure to GB Vapor in Air:
Diagnosis/Dosimetry, Lowest Observable Effect Levels,
Performance-Incapacitation, and Possible Delayed Effets

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13. ABSTRACT Purpose: establish for both vehicle-pretreated and pyridostigmine-pretreated conscious guinea pigs and marmosets whole-body exposed to low levels of GB vapor: (i) The *Lowest Observable Effect Level (LOEL)* for GB, i.e., the C.t-value (t = 5 h) of exposure at which an internal dose (fluoride-regenerated GB from blood BuChE) becomes measurable; (ii) The *Lowest Observable Adverse Effect Level (LOAEL)* for GB, i.e., the C.t-value (t = 5 h) of exposure at which effects start to decrease performance; (iii) Whether unexpected adverse effects on performance emerge through the combination of pyridostigmine-pretreatment and GB exposure. A validated system was developed for generating, analyzing semi-continuously, and exposing conscious animals to low levels (8-80 ppt: 0.05-0.5 $\mu g/m^3$) of GB and other nerve agents. Read-out parameters reflecting adverse effects: pupil size (miosis), EEG, visual evoked response (VER) and startle-response for both species, and shuttle-box active avoidance behavior for guinea pigs, and the bungalow-test for marmosets. *LOEL* and miosis were investigated most thoroughly whereas changes in the other parameters should be considered indicative for incapacitation and require further investigation. The lowest exposure concentration of GB which resulted in significant ($p < 0.05$) effects on these parameters compared to air exposure, was taken for calculating the *LOAEL* (C.t-value) for each of these parameters.

LOEL (mg.min.m^{-3})			
Guinea pig		Marmoset	
Vehicle	Pyrido	Vehicle	Pyrido
0.010 ± 0.002	0.014 ± **0.003**	0.04 ± **0.01**	0.048 ± **0.002**

	LOAEL (mg.min.m^{-3})			
	Guinea Pig		Marmoset	
Read-out parameters	Vehicle	Pyrido	Vehicle	Pyrido
Miosis	1.8 ± 0.3	1.8 ± 0.5	2.5 ± 0.8	3.0 ± 0.8
	LOAEL (mg.min.m^{-3})			
EEG	0.8	0.4	0.2	0.1
VER	0.8	0.8	25	4.4
Startle-response	> 44	15.2	6.5	4.4
Shuttle-box behavior	2.1	60	-	-
Bungalow-test	-	-	14.9	7.2

These data were addressed in the light of the recommended occupational and detection limits for GB vapor in air.

14. SUBJECT TERMS
Gulf War, Sarin (GB), Low Level, Diagnosis, Dosimetry, LOEL, LOAEL, Performance-Incapacitation, Guinea pig, Marmoset

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INTRODUCTION

Many attempts have been made to define various degrees of low level exposure. Rather than adhering to one of these definitions, our choice was to expose animals to the lowest controllable concentrations of agent and to increase these concentrations untill effects become visible. Previous work on low level exposure to CW-agents related to environmental conditions (occupational exposure) during planned destruction of these agents (McNamara and Leitnaker 1971). Various developments lead to the notion that the effects of low level exposure to chemical warfare agents on military personnel become also increasingly important under actual battlefield conditions. Several realistic circumstances can be envisaged where low level exposure may take place: (1) Small amounts of agent may penetrate through the closures and through unnoticed slight damages to protecting clothing or gas masks. (2) Imperfections during donning and doffing procedures of protective gear will have the same effect. (3) Personnel performing duty in collectively protected areas may be exposed to small amounts of agent due to entry and exit procedures of the area and residual contamination of entering personnel. (4) Both offgassing and the physical contact with decontaminated material (painted surfaces, protective clothing) may contribute to low level exposure. (5) Possible exposure of personnel due to a downwind transport of an agent over long distances from contaminated areas, e.g., due to destruction of enemy stockpiles (suggested as a possible contributing factor to the Gulf War Syndrome; Ember 1996).

The possibility that military personnel may be low level exposed to chemical warfare agents in a conflict situation not only makes it necessary to ascertain that exposure has taken place based on detection and reliable diagnosis/dosimetry of trace exposure, but also to establish at which vapor concentrations in air, minimal (systemic) effects will become observable (as for occupational exposure), but also at which concentrations these effects start to have adverse effects on performance.

In a recent publication on Airborne Exposure Limits for G-agents, the Research and Technology Directorate of Edgewood Research, Development & Engineering Center (ERDEC) recommended limits for occupational exposure, i.e., for workers without respiratory protection: a maximum averaged air concentration of 0.0001 mg/m^3, now referred to as the "Worker Population Limit (WPL) (averaged over an eight hour work day) (Mioduszewski et al 1998) (see Appendix). This would correspond with a no-effect level (C.t-value) of 0.048 $mg.min.m^{-3}$. At this occupational limit, even the mildest miosis or cholinesterase depression should not occur. The acute exposure limits for occupational workers, i.e., those for Immediately Dangerous to Life or Health (IDLH), for a 30 min exposure to GB, is 0.1 mg/m^3. The Short-Term Exposure Limit (STEL) for GB limited to 15 min for up to 4 times in an 8 hour work day, is 0.002 mg/m^3. Likewise, the acute exposure guideline levels for the general population for exposure durations of (a) 30 min: 0.0024 mg/m^3, (b) 1 hour: 0.0012 mg/m^3, and 4 hours: 0.0003 mg/m^3.
For several reasons we have chosen sarin (GB) as the target compound for the study of effects of nerve agents during low level exposure: (1) several potential adversaries have stockpiled this agent for possible use in chemical warfare; (2) it has been claimed that US soldiers may have been exposed to sarin during or in the aftermath of the Gulf War when rockets filled with GB were destroyed near Bunker 73 at Khamisiyah, Iraq (Ember 1996); (3) GB has been used in terrorist attacks by the AUM Shinriyko sect in Matsumoto (1994) and Tokyo (1995) (Croddy 1995, and Polhuijs et al 1997).

The ongoing controversy and discussions whether during the Gulf War soldiers have been low level exposed to chemical warfare agents such as sarin and the hypothesis that delayed effects of nerve agent exposure may be due to "even imperceptible" poisoning (SIPRI 1975), show clearly that drastic improvement with regard to sensitivity and reliability are needed for diagnosis/internal dosimetry of exposure to CW-agents. If such improved methodology is not available, it will not be possible to resolve the key question whether observed or alleged late effects can possibly be ascribed to exposure to nerve agents, and to which dose of the agent. We therefore proposed to use a method to establish the internal dose, based on the release of the inhibitor phosphyl moiety from butyrylcholinesterase (BuChE) in plasma with fluoride ions followed by GC analysis of reconstituted GB. Releasing methylphosphonic acid from the aged enzyme and subsequent derivatization for GC analysis is still under development. Recently, we applied this procedure to serum samples from victims of the terrorist attacks with nerve agents in the Tokyo subway and in Matsumoto (Polhuijs *et al.* 1997). We estimate that 0.01 % BuChE inhibition can be measured in this way, i.e., an approximately 1000-fold improvement over previous methodology based on measurement of blood cholinesterase (ChE) activity. Moreover, using this new method cumulative exposure over several weeks can be measured.
We consider the measurement of the internal dose as essential for the determination of exposure levels (C.t) that correspond with the lowest observable effect level *(LOEL)* in guinea pigs and in marmoset monkeys, i.e., the C.t level at which GB starts to penetrate into the systemic circulation. Furthermore, the exposure levels and internal doses can be determined at which adverse effects on performance become manifest.

In our view an additional aspect should be addressed, in particular in the case of GB. Like in the Gulf War, military personnel will be either pyridostigmine-pretreated or not, depending on risk assessments.
In view of the above-mentioned arguments the following questions were addressed for both groups of soldiers in the present investigation:

(1) At which C.t-value ($t \leq 5$ h) of GB exposure does an internal dose become measurable in blood, i.e., what is the *Lowest Observable Effect Level (LOEL)* of Exposure?
(2) At which C.t-value of GB exposure and internal dose do (systemic) effects of exposure start to have adverse effects on the performance of military personnel, i.e., what is the *Lowest Observable Adverse Effect Level (LOAEL)* of Exposure?
(3) What are the consequences of continuous pretreatment during low level exposure to GB, i.e., will unexpected adverse effects on performance emerge through this combination of two ChE-inhibitors?

Read-out parameters reflecting adverse effects on performance during whole-body exposure to low levels of GB vapor were pupil size (miosis), electroencephalogram (EEG), and visual evoked response (VER). Parameters measured at 1-1.5 h after exposure were startle-response (for guinea pigs and marmosets), shuttle-box active avoidance behavior (for guinea pigs), and locomotor activity (bungalow-test) for marmosets. It should be emphasized that the *LOEL* of exposure and miosis were most thoroughly investigated in the present study. It should further be emphasized that any effect on all these parameters should be significantly different ($p < 0.05$) from corresponding effects in air-exposed animals to be considered as an adverse effect directly or indirectly attributible to GB. The lowest exposure concentration of GB which resulted in significant effects on these parameters during a 5 h exposure was chosen for calculating the *LOAEL* (C.t-value) for each of these parameters.

MATERIALS AND METHODS

In general

In this study about one year was allocated for development of exposure equipment and tightening up of the available analytical techniques for measuring low levels of GB. First of all the *LOEL* had to be assessed. This level was defined as the lowest measurable internal concentration of GB bound to butyrylcholinesterase (BuChE) and other binding sites such as carboxylesterase (CaE) in blood that could be measured in guinea pigs and marmosets during a 5 h exposure period. The first GB exposures of guinea pigs to approximately 40 $\mu g/m^3$ GB were performed in the fall of 1998 (Van Helden et al 1998). It soon became clear that at this relatively low vapor concentration of GB the fluoride-regenerated GB was distinctly measurable in blood within the first 30 – 60 min of exposure while the animals showed already miosis. It was even unclear at that time, whether miosis or the internal dose of GB in blood was the first measurable parameter. These early findings forced us to find out how to generate much lower GB vapor concentrations, how to expose animals to these concentrations, and how to analyse such low concentrations. The judgement at that moment was that we should be able to generate a 100-fold lower vapor concentration of GB. Generating such low concentrations of GB was associated with technical problems, not only in view of the vapor generation of GB itself, but also from an analytical point of view and even regarding the exposure of animals in a reliable way. We ultimately succeeded in resolving these technical problems and are now able to generate, to expose animals to, and to measure concentrations of GB semi-continuously (every 2-5 min) in the range of 0.05 – 1.0 $\mu g/m^3$ (8 –160 ppt) (see Results).

Animals

Male Dunkin-Hartley albino guinea pigs [HSD-Harlan (Harlan)] with a starting body weight of 350-400 g were used. The animals were kept 3 to a cage and the ambient temperature was regulated at 20-22 °C. After surgery for placing EEG-electrodes or Alzet osmotic pumps (see below), the animals were housed individually. Relative humidity was monitored but not regulated and was always found to be higher than 50%.

Marmosets (Callithrix jacchus) (Harlan, UK) with a body weight between 200 – 420 g were used. The protocols for the animal experiments were approved by the TNO Committee on Animal Care and Use.

Equipment for generating, analyzing and exposing guinea pigs and marmosets to GB

In order to analyze GB vapor containing 0.05 – 1.0 $\mu g/m^3$ (8 –160 ppt) semi-continuously, i.e., at 2-5 min intervals, the use of pre-concentration techniques such as "cold-trapping" is essential. Semi-continuous measurements were necessary since we found that large fluctuations of the concentrations of GB in air occur at trace levels due to adsorption/decomposition on the skin, urine and faeces of the animal. A complete equipment for analysis of GB vapor in air at levels ≥ 0.05 $\mu g/m^3$ (8 ppt) semi-continuously, consisting of a gas sampling valve with 'cold loop' connected to a GC with a nitrogen phosphorus detector (NP) is now used routinely. A time based average of all the measured vapor concentrations was used to calculate the mean concentration over a particular period of exposure time. See Fig 1 for a schematic representation of the equipment for vapor generation, exposure and analysis. For an extensive updating of the system and validation of the analysis of GB, see Results.

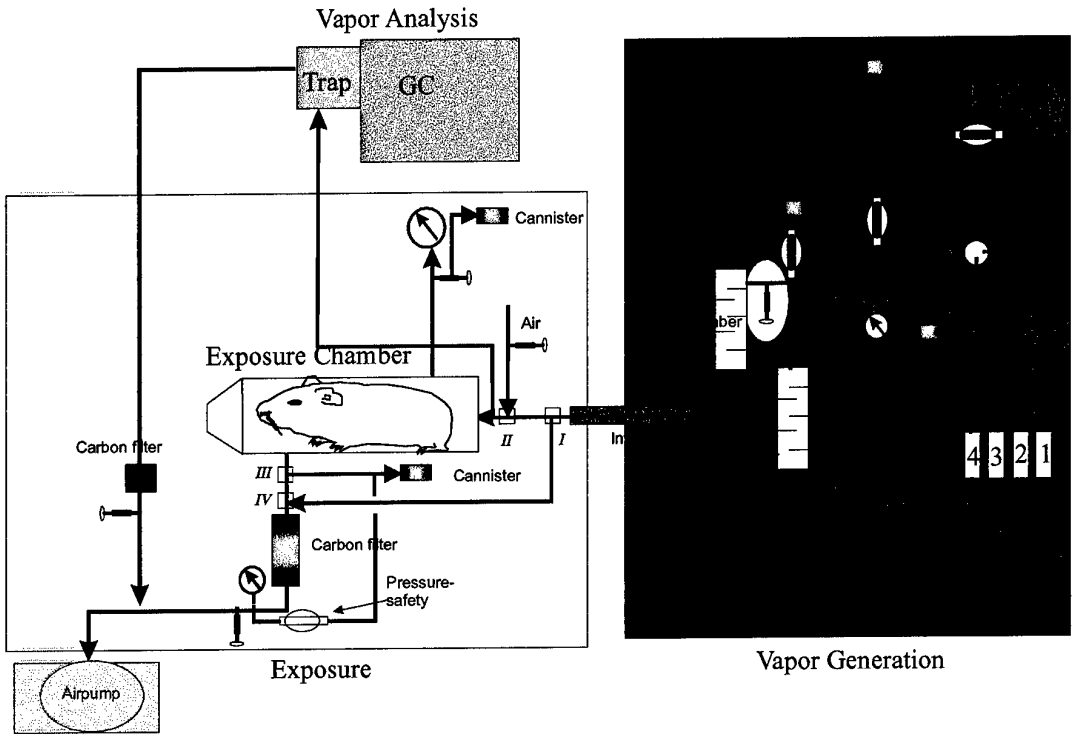

Fig 1. Schematic diagram of equipment for vapor generation (right panel), exposure (left panel) and analysis (upper small panel). To generate the required very low concentrations of GB vapor in air, mass flow controllers (no. 1-4), gas mixing chambers, cannisters, a cooling bath and valves are needed. The GB vapor is transported through a heated interface and routed to the animal exposure chamber. Vapor samples for analysis are taken just before the animal exposure chamber, i.e., close to the animal. The samples are then transported to the Cold Sample Trap (CST) for sample enrichment after which the sample is directly injected into the GC for analysis. The combination of CST with the selective and sensitive NP detector of the GC allow us to detect GB at the required ppt levels. I – IV are three-way valves.

Fluoride-induced reactivation of sarin-inhibited esterases in blood

Heparinized blood, taken from the guinea pigs and marmosets via the carotid canulla, was stored on ice. After the blood volume was determined, the sample was centrifuged for 4 min at 14000 rpm at ambient temperature. The upper layer, i.e., plasma, was diluted with three parts of an acidic buffer solution (0.189 M acetic acid and 10.8 mM sodium acetate trihydrate; pH 3.5) resulting in a reaction mixture with a pH of approx. 4. The mixture was incubated with potassium fluoride to a final concentration of 250 mM for 15 min at 25°C. In case of the LOEL assessment for marmosets, blood samples were directly used instead of plasma because otherwise the sample volume was too low (approx. 50-100 µl).

Solid-phase extraction of sarin

Sarin (GB) was isolated from the acidic mixture by means of solid phase extraction. The SepPak C_{18} cartridge (type 'Classic',Waters, Milford, MA, USA) was preconditioned with 4 ml of ethyl acetate and two times 5 ml of water. A 25 µl sample of an internal standard solution (deuterated sarin, d7-GB, in ethyl acetate) was added to the sample mixture. Next, the mixture was charged onto the cartridge. The cartridge was washed with 5 ml of water and

dried with air. After adding 1.2 ml of ethyl acetate, sarin with its internal standard were eluted from the cartridge. Small droplets of water which coelute from the cartridge were seperated from the ethyl acetate layer by freezing off the water by placing the vial containing this mixture in a dry ice/acetone bath. The ethyl acetate phase (approx. 600-800 µl) was stored in GC vials at -20°C until analysis.

Gaschromatographic analysis of GB

Sample loading

The sample was charged in portions of 100 µl onto a pre-conditioned glass TCT sample tube filled with ca. 100 mg of Tenax TA (60-80 mesh) and blown dry with a flow (250 ml/min) of nitrogen. For large samples, i.e., up to 500 µl, the tube was purged for 45 minutes. Next, the tube with sample was placed in the TCT injection unit and the system was started.

GB-analysis in blood samples

The GB-analysis was performed by means of two-dimentional chromatography with large volume injection, as described by Van Helden et al (1998).

Briefly, a Carlo Erba (Milano, Italy) 5300 Mega series Gas chromatograph was equipped with a flame ionisation detector (FID) and a nitrogen phosphorus detector (NPD), a Chrompack (Middelburg, The Netherlands) MUSIC (Multiple Switching Intelligent Controller) and a Chrompack TCT (Thermal Cold Trap) injector (see Fig 2). Flow rates of air and hydrogen through the FID and the NPD were 350 and 35 ml/min respectively. Helium was used as make-up flow (38 ml/min) for the NPD. The temperature of the detector bases was set at 250 °C, whereas the temperature of the injector base was set at 200 °C.

A CP Sil 8 CB column (length, 30 m; i.d., 0.53 mm; film thickness 5 µm) was used as a pre-column and a CP Sil 19 CB column (length, 60 m; i.d., 0.32 mm; film thickness 1 µm) was used as the analytical column for analysis of GB in plasma during *LOEL* and *LOAEL* experiments with GB in the guinea pig. Subsequently, the system was modified for more selectivity and to make it more robust. The analytical column was replaced by a CP Wax 57 column (length, 30 m; i.d., 0.25 mm; film thickness 0.5 µm). The TCT trap consists of a medium polar deactivated retention gap (i.d. 0.53 mm). All columns were purchased from Chrompack Int. (Middelburg, The Netherlands).

Data acquisition was performed with a data acquisition software program Harley Systems Peak Master ™ ('s Hertogenbosch, The Netherlands) which runs on a PC.

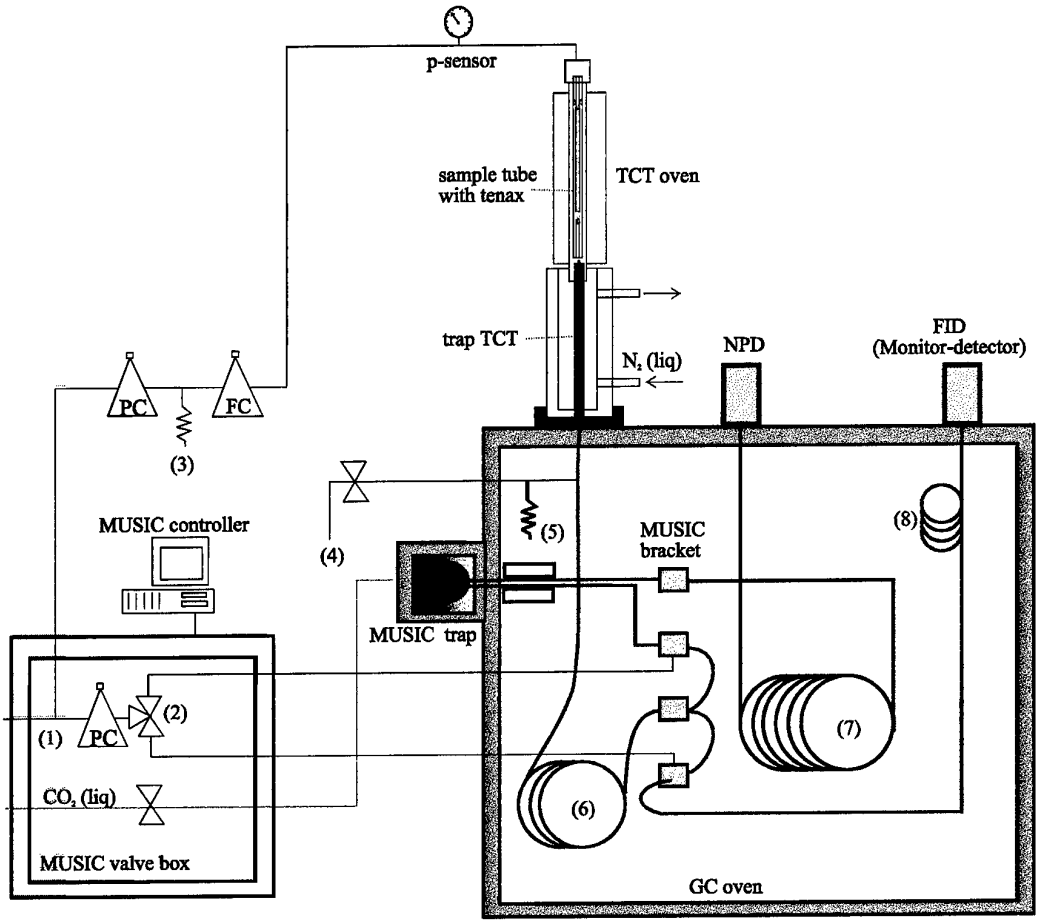

Fig 2. Schematic representation of the GC configuration used for GB trace analysis (TCT-MUSIC-NPD): (1) helium carrier gas, (2) Deans switch for pressure switching, (3) purge flow carrier, (4) desorption flow, (5) purge flow, (6) pre-column, (7) analytical column, (8) restriction. For further description see text.

Description of GB analysis on the modified TCT-MUSIC system

Helium (see Fig 2) gas is used as carrier (1), and pressure regulated (110 kPa) for the analytical column. The pre-column is flow regulated (approx. 16 ml/min). By putting a pressure regulator and installing a purge flow (3) of approx. 30 ml/min before the flow regulator, contamination of carrier gas is prevented. In trace analysis blank analyses are essential, and in this way guaranteed. The maximum pressure for maintaining the flow is set at 2.5 bar, while the main pressure is set at 5 bar. In this way, any blocking of the flow will prevent back diffusion into the main system.

The glass tube filled with appr. 100 mg Tenax TA (60-80 mesh) and loaded with sample is placed in the TCT oven. The oven is heated for 6 min at 180 °C. During desorption, valve (4) is opened and the TCT trap is cooled with liquid nitrogen (-90°C). The fraction of interest is collected on this trap and re-injected onto the pre-column (6) by flash-heating the trap (12 °C/min) from –90 °C to 200 °C (12 min). During and after the re-injection the desorption flow will be closed. The gas line with rests of ethyl acetate is purged with a low helium flow by installing a restriction capillary (5) of 1 m (50 µm i.d.), placed in the oven to prevent clogging. This improves the pre-column injection chromatogram considerably. During pre-

column analysis the oven is kept contant at 70°C for 6 min and programmed to 90°C at a rate of 10°C/min.The MUSIC controller and valve box are set for trapping the GB and d7-GB fraction (7.1 - 8 min) from the effluent of the pre-column (6) by 'Deans swiching' with valve (2). The fraction is collected at –70 °C in the MUSIC trap and re-injected on the analytical column after cooling down the GC oven to 70°C. The temperature of the oven is programmed from 70°C to 220°C at a rate of 5°C/min and kept constant for 15 minutes. The d7-GB and GB are seperated and detected with the NPD. The retention time for d7-GB and GB are respectively 10:35 min and 10:46 min with a resolution (R_s) of 1.9. The absolute detection limit (S/N = 2) for GB was approx. 0.15 pg depending on the noise (500 µl injection of 0.3 pg/ml GB in ethyl acetate). A typical example of a GB chromatogram is given in Fig 3.

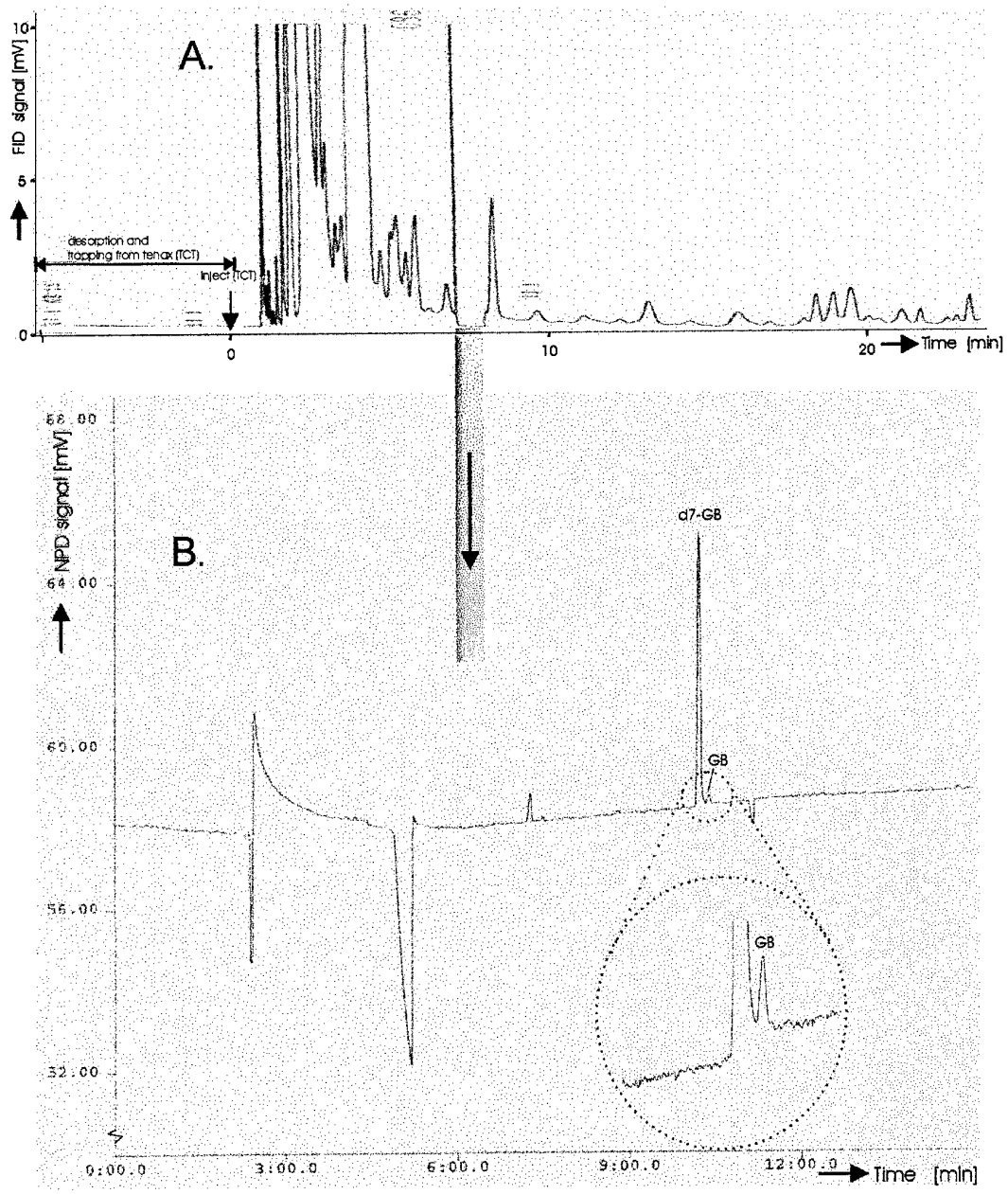

Fig 3. Representative GC injection (500 µl) of a plasma extract in ethyl acetate. The sample was taken from a pyridostigmine-pretreated marmoset (M8) at t = 300 min of exposure to GB (0.4 µg/m³). Two-dimensional analysis: A) pre-column chromatogram, and B) analytical column chromatogram of the re-injected fraction (arrow in color). The analyzed GB concentration was 12 pg/ml plasma (S/N= 8).

Special precautions for trace analysis

The GC-configuration was tested for carry-over (memory effect). There seemed to be no carry-over in the concentration range applied for the experiments. Calibration of the system was performed on a daily basis. The response factor was stable during the various experiments of the guinea pigs and marmosets. In this period of several months the variation coefficient was less than 10%. The calibration curve was linear in the analyzed concentration range (corr. coef. > 0.999), also with a relatively large amount of internal standard added to the samples (155 pg d7-GB). To check the sample work-up, guinea pig blood was spiked with

GB (188 pg/ml) and worked up as described before. The overall recovery was 67% relative to the internal standard.

Before analyzing sample extracts, a blanc solvent was injected and checked for interfering peaks. In order to prevent any carry-over by Tenax tubes, these tubes were washed with a few ml ethyl acetate and heated under a stream of helium (≥ 14 h, 220 °C). After this cleaning step, the tubes were capped. The extracts were stored at –20 °C separated from standards. Also the internal standard solution was stored seperately to prevent contamination.

Assessment of the LOEL for GB exposure
In order to determine the *LOEL* for vehicle (n = 6: Gp13-18)- or pyridostigmine-pretreated (n = 6: Gp19-24) guinea pigs and for vehicle-pretreated marmosets (n = 5: M1-5), or pyridostigmine-pretreated marmosets (n = 5: M6-10), Alzet osmotic minipumps (Model 2002 Alza Corp., Palo Alto, CA) containing either vehicle (20% propylene glycol, 10% ethanol, 70% water (1 part glacial acetic acid in 2000 parts distilled water) or pyridostigmine (0.04 mg/kg/h and 0.02 mg/kg/h, respectively) were implanted subcutaneously under halothane N_2O anaesthesia 4 days before the GB exposure started (For further details, see Van Helden et al 1998). One animal per day was exposed to 0.05 – 0.6 $\mu g/m^3$ GB during a period of 5 h. During exposure blood samples (500 – 700 µl) were taken every 30 min for internal dose assessment in guinea pigs. From marmosets 200 –300 µl blood samples were drawn.

Assessment of the LOAEL for GB exposure
Experimental procedure for guinea pigs
To determine the *LOAELs* for GB exposure regarding miosis, EEG, VER, startle-response and shuttle-box behavior during GB exposure for vehicle or pyridostigmine-pretreated guinea pigs, four groups of restrained conscious animals were used:

1. Vehicle-pretreated guinea pigs (n = 6: Gp25-30) were exposed to air for 5 h (base-line controls).
2. Vehicle-pretreated guinea pigs (n = 12: Gp49-54 and Gp61-66) were exposed to approximately 7.5, 15, 25, 50 or 150 $\mu g/m^3$ GB for 5 h. Two animals were exposed at a time, one of them was used to take blood samples from, the other one for measuring miosis, EEG/VER during exposure, and for performance-testing (startle-response, shuttle-box) at the end of the 5 h exposure period. Gp49-54 were used for taking blood samples.
3. Pyridostigmine-pretreated guinea pigs (n = 6: Gp31-36) were exposed to air for 5 h (base-line controls).
4. Pyridostigmine-pretreated guinea pigs (n = 12: Gp55-60 and Gp67-72) were exposed to approximately 11.5, 15, 25, 50, 150 or 200 $\mu g.m^{-3}$ GB for 5 h. Two animals were exposed at a time, one of them to take blood samples from every 30 min, the other one for measuring miosis, EEG/VER during exposure, and for performance-testing (startle-response, shuttle-box) 1-1.5 h after the end of the 5 h exposure period. Gp55-60 were used for taking blood samples.

Experimental procedure for marmosets
To determine the *LOAELs* for GB exposure regarding miosis, EEG, VER, startle-response and bungalow-test behavior for vehicle or pyridostigmine-pretreated marmosets, two groups, each consisting of 5 restrained conscious animals, were used:

1. Vehicle-pretreated marmosets (n = 5: M11-15) were exposed to air for 5 h (base-line controls).

Two days later, these vehicle-pretreated marmosets were exposed to approximately 7.5, 15, 25, 50 or 150 $\mu g/m^3$ GB (n = 1 per concentration) for 5 h.

2. Pyridostigmine-pretreated marmosets (n = 5: M16-20) were exposed to air for 5 h (baseline controls). Two days later, these pyridostigmine-pretreated marmosets were exposed to approximately 7.5, 15, 25, 50, 150 $\mu g/m^3$ GB (n = 1 per concentration) for 5 h.

During exposure to air or GB, pupil diameter (miosis), EEG, and VER were measured, 1-1.5 h after exposure startle-response and bungalow-test behavior were determined.

In general

Three weeks in advance of the air or GB exposure of the guinea pigs, training for the shuttle-box performance started. Alzet pumps containing vehicle or pyridostigmine (0.04 mg/kg/h) were placed subcutaneously 4 days before the exposures started. Marmosets should not be trained for their bungalow-test and received Alzet pumps containing vehicle or pyridostigmine (0.02 mg/kg/h) subcutaneously 4 days before the exposure started. It was established earlier for both species that 30% of blood ChE-inhibition caused by pyridostigmine was stable 4 days after the insertion of the Alzet pump.

As mentioned, (1) miosis, (2) EEG and (3) VER were measured online during exposure, whereas blood samples were taken every 30 min for the assessment of internal dose of GB and AChE-activity afterwards. After the 5 h exposure period the animals were degassed in an animal cage wrapped in a piece of protective military clothing to absorb GB. This cage containing the animal was put in a laminar flow chamber for 1 h. Next, (4) startle-response (guinea pigs and marmosets), (5) shuttle-box behavior (guinea pigs), and (6) bungalow-behavior (marmosets) were tested at 1-1.5 h after exposure (see below).

1. Assessment of the pupil size (miosis)

Photographs were taken from both eyes every 10 min using two digital cameras (Sony, types MVC-FD-7), each on a support and directed to one of the eyes. Advantageously, taking digital pictures instead of conventional ones permits their quality control online and the picture data can be stored on CD-rom. The ultimate choice of a camera was hampered by the fact that photographs should be taken under dimlight conditions, i.e., 100 Lux light intensity. This light intensity was measured by a Lux-meter (Elvos LM-1010 Luxmesser) and was kept constant during the 5 h exposure period. Dimlight conditions were necessary to have a large pupil size (i.e., mydriasis) at the start of the GB exposure. In contrast to conventional cameras digital cameras are able to take photographs at 100 Lux. Furthermore we adjusted the digital cameras in a way to enable flashing with an external flasher which could also be used to induce the visual evoked response (VER) in the EEG signal. Finally, using photoprogram software such as Photo Express and Microsoft Photo Editor, the diameters of the iris and pupil could be measured accurately (Van Helden et al 1998). For a typical example, see Fig 4.

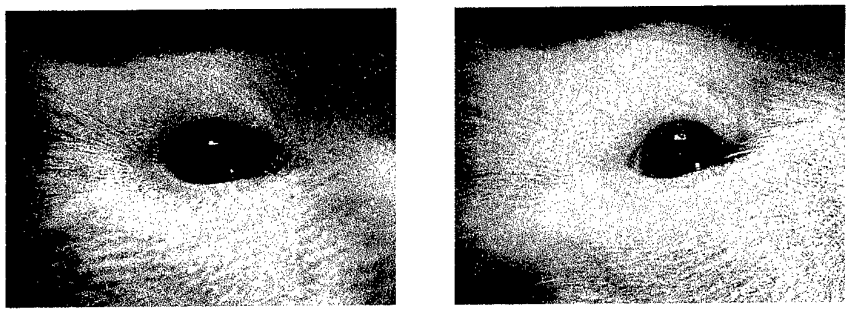

Fig 4. Typical example of guinea pig eyes demonstrating the pupil and iris diameters before (left panel) and after GB exposure (right panel).

2. Assessment of EEG by telemetry

Each animal was provided with a transmitter (TL10M1-F50-W) (Data Sciences International, Minnesota, USA), while under halothane/N_2O anesthesia, for telemetric monitoring of EEG and VER-potentials. The transmitter was directly connected to the EEG electrodes implanted through the skull on the dura mater; bipolar recordings were taken from the visual cortex region (area 17). A general receiver converts telemetered data to a form readily accessible by a PCL812 AD card (ADVANTECH). Collection and analysis of telemetered data was performed by a PC. The EEG signals were amplified (50.000x), filtered (0.3-30 Hz) and fed into the ADC of a PC. During a 5-h exposure to GB the EEG was routinely evaluated on-line by visual inspection. To avoid subjective bias and to permit a quantitative analysis, 5 epochs of 10 sec were chosen from a total recording period of 200 sec. EEG data were also preprocessed by spectral analysis using the Fast Fourier Transformation (FFT) technique. FFT spectra were averaged per animal for statistical analysis. This analysis determines the EEG energies in each of the classical EEG frequency bands: Delta (d)1 = 0.8-2.0 Hz, Delta 2 = 2.0-3.5, Theta (t)1 = 3.5-5.5 Hz, Theta 2 = 5.5-7.5 Hz, Alpha (a)1 = 7.5-10 Hz, Alpha 2 =10-12.5, Beta (b)1 = 12.5-18 Hz, Beta 2 = 18-25 Hz. The total power (V^2) of the various frequency classes was used for the evaluation of the electrical brain activity.

3. Assessment of visual evoked response (VER)

VERs were elicited by 30 light flashes provided by a Xenon flasher at a time interval of 2 s ± 20%. These very short light flashes did not influence miosis. The signals were amplified (50.000x), filtered (0.1-500 Hz), fed into the ADC of a PC and averaged (n = 30). The sampling rate used was 1 kHz. Latency and/or amplitudes of the positive and negative peaks were determined per animal; subsequently a grand average of the VERs of all animals was made and compared with the baseline values according to Wolthuis *et al* (1991). For the guinea pigs the latency parameters t_1, t_2, t_3 and t_4 (Fig 5) were analysed, for the marmosets only t_2 and t_3 were analyzed, because the VER-signal from the marmoset differs from that of the guinea pig. For statistical analysis, see below.

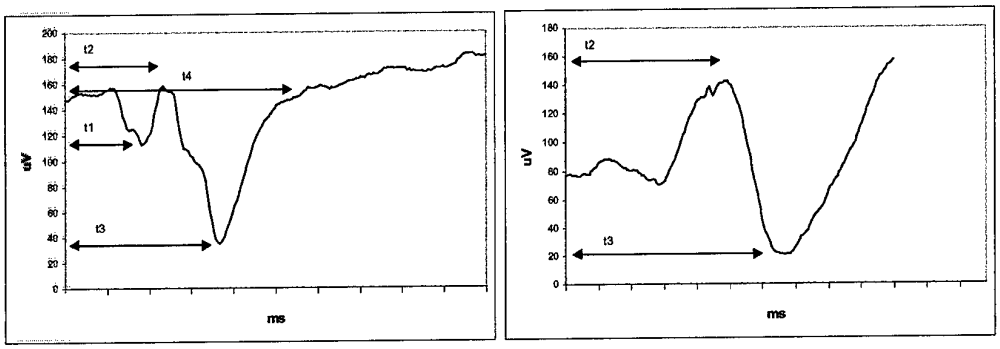

Fig 5. An example of a VER signal of a guinea pig (left panel) and that of a marmoset (right panel), received by telemetry in which the latency parameters to be measured, i.e., t_1, t_2, t_3 and t_4 are indicated.

4. Assessment of startle-response

In this test the stretching movement of the legs is used to reflect the reaction of the animal on a startle signal. While standing on their hind paws, immobilized in a vertically mounted PVC tube (for guinea pigs: 7 cm diameter, 16.5 cm height; for marmosets: in a box, 17.5 cm in diameter, 26 cm length), the animals were exposed to 20 sound stimuli (for guinea pigs:120 db, 10 kHz, "white noise", 20 ms; for marmosets: 120 db, "pink noise", 20 ms) (see Fig 6). Such a stimulus leads to a reflex extension of the limbs (startle response). The response of the hind limbs is recorded by a force transducer connected to the platform on which the animal is standing. The responses are fed into the ADC of a PC and averaged on line. The amplitude, or total area under the curve and the latency of the Auditory Startle Reflex were determined according to Philippens *et al* (1996) and were used to express the motor reaction of the startle reflex.

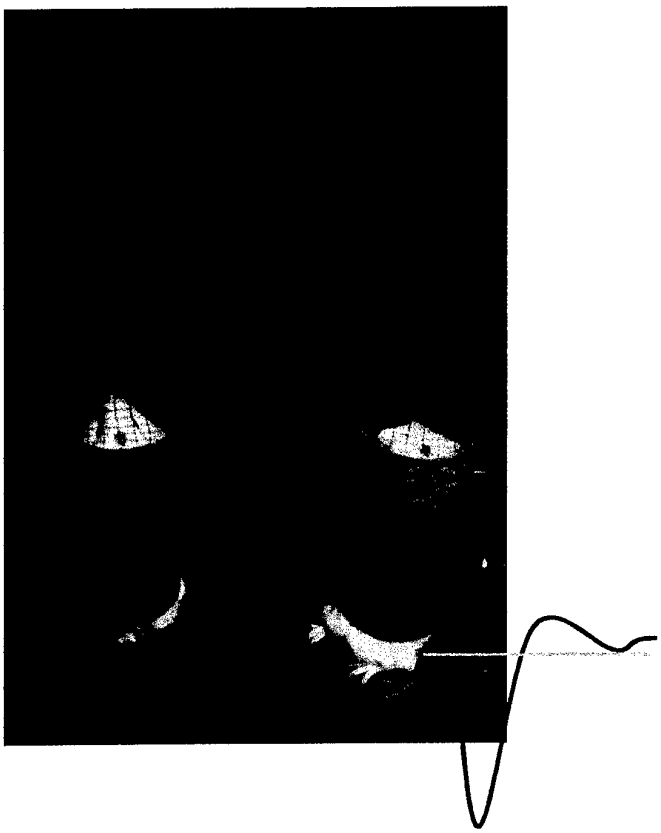

Fig 6. Experimental setup to determine an auditory startle-response of 200 ms duration (auditory startle pulse: 20 ms, 120 dB, 10 kHz). The motor response of the guinea pigs standing on a platform is registered by a connected force transducer and transferred to a computer. Parameters are amplitude and area under the curve of the startle response.

5. Assessment of active avoidance behavior (shuttle-box)

In this test the active avoidance of an unpleasant event upon a conditioned stimulus (CS) is used to measure the retrieval of learned behavior. The active avoidance technique applied was an automated two-way shuttle-box, using a sound signal as the conditioned (CS) and a stream of air (about 6 L/s, air tube diameter 1 cm) as an unconditioned (UCS) stimulus. The shuttle-box consists of two equal compartments (23x23x23 cm) with rounded corners, connected by a gate provided with an infrared beam detector, through which the animal may cross from one compartment to the other. Per day, each animal received one training session of 20 trials, during which the animal had to learn to avoid the UCS by moving into the other compartment within 10 s after the sound signal had been turned on. The sound signal stops when the guinea pig has passed through the gate. When the animal fails to avoid, the stream of air is directed into the compartment in which the animal is present is turned on and stops when the animal has escaped into the other compartment. The intertrial interval is 20-30 s. After 12 animals had reached their criterion, which was 80% or more correct responses, Alzet pumps containing vehicle (n = 6) or pyridostigmine (n = 6) were installed subcutaneously. Four days later all animals were tested again in the shuttle-box to obtain base-line values, after which the GB exposures started. The number of correct avoidance reactions (CAR) were used to measure effects on learned behavior.

6. Assessment of bungalow-test behavior

This is an automated test to measure exploratory and motor activity of marmosets (Wolthuis *et al* 1994). The apparatus consists of four horizontally placed non-transparent PVC compartments (25x25x25 cm) with a meshwire top, interconnected by PVC tubes (inner diameter 9.5 cm), resembling a four-room bungalow. The tubes are wide enough to allow the animal to move to each of the three other compartments. The compartments are placed in a square and the distance (heart to heart) of the compartments to the adjacent ones is 43 cm. Four lights are mounted on the closed ceiling of the apparatus. The floors of the compartments are made of white plastic and reflect light. On each of the meshwire tops a photocell is mounted that is linked to an IBM-compatible PC. A TV-camera is mounted. The bottom of each compartment reflects the light that is registered by the photocells. The presence of a marmoset in a compartment is detected by a photocell. Testing of an animal lasts 20 min. Control measurements are performed twice; the results of the second control test are taken as the starting value for each animal. Software was developed that allows automated registration of: (a) time spent and time intervals of the presence of the animal in each box, (b) the number of times that the animal switches from one box to another, and (c) from which box these switches take place. For statistical comparisons, the multiple t-test of Welch (Natrella *et al* 1963) is applied. The number of compartment changes was used to measure effects on the spontaneous locomotor behavior.

Determination of AChE-activity in blood

Blood samples (5 µl) obtained online every 30 min during exposure, were immediately mixed with 1% saponin (BDH, Poole, UK), frozen in liquid nitrogen and stored at -70°C. After appropriate dilution, AChE-activity was assessed using a radiometric method (Johnson and Russell, 1975). The final concentration of ACh was 12 µM; 3H-ACh iodide (NEN, Dreiech, Germany) was diluted to a specific activity of 602 MBq.mmol^{-1}. Ethopropazine (2.5 µM, St.Louis, Mo, USA) was used as a specific inhibitor of BuChE. Electric eel AChE was used as a reference.

Restrainment of the animals

A special guinea pig restraint was necessary to restrain the animal properly in order to take photographs of both eyes during exposure and to take blood samples from the carotid (left) cannula as described by Van Helden et al (1998). Briefly, a metal tube-like grid to enclose the animal was mounted on a floor grid. The use of a metal neck-bow as well as a "jaw-bone print", made of synthetic material, were necessary to optimize restrainment of the animal. We also built a sort of "bunk bed" to expose two animals at a time; one animal to take blood samples from, via a carotid cannula, the other to be used for miosis, EEG/VER and performance testing after the 5 h exposure to GB or air (control).

The conscious marmoset is seated in a special metal chair (Fig 7), his arms and legs fixed on the chair, wearing a plastic helmet in order to fix the animals head to the chair in order to take photographs from both eyes every 10 min using a digital camera on a stand. The animals have learned to sit in this way in the exposure chamber for several hours while whatching video-films about marmosets. The video-film appeared to be necesarry to keep the animals awake.

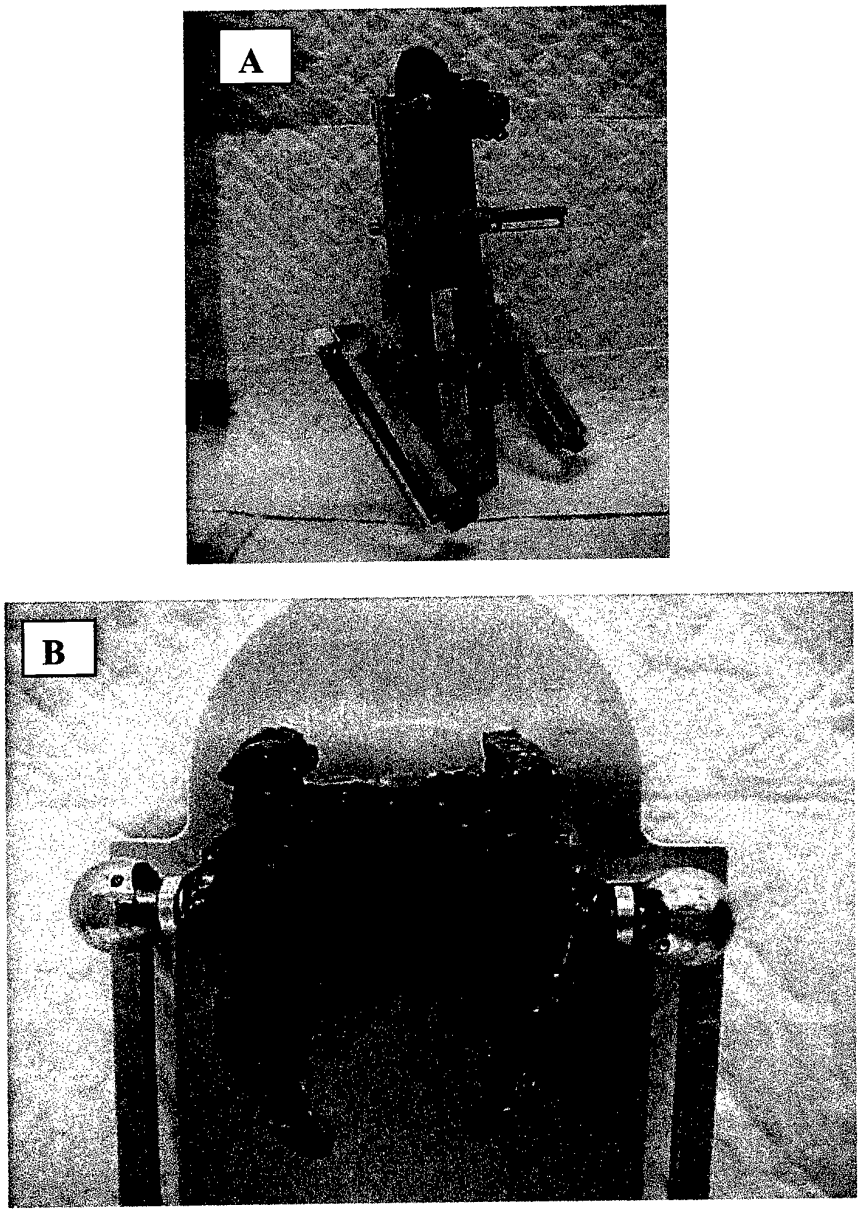

Fig 7. Special marmoset chair (panel A) and detail of helmet (panel B) to fix its head.

Statistical analysis

For statistical analysis the TNO Department for Applied Statistics (Head: Dr P Defize) was consulted. To compare the read-out parameters (blood AChE-activity, miosis, EEG, VER, startle-response and shuttle-box behavior), obtained per animal exposed to GB, with the averaged values of the corresponding parameters obtained from six air-exposed animals (controls), a two-sample t-test was used under the assumption that the variance in both the control group (n = 6) and the experimental group (n = 1) was equivalent (Montgomery 1991). The following equations were used: s**2 = SUM(xi - MEAN(x))/(n-1), and t = (y-MEAN(x))/(s*SQRT(1+1/n)), in which x = control value, y = value to be tested against control. The energies of the various EEG-bands at set time intervals were standardized as follows before statistical analysis: let A and B be the EEG-band energy within an animal at t = 0 and t = 30 min, respectively. Then standardized band-energy at t = 30 was set to A/B+B/A. The same standardized calculations were done at t = 60, 90 min. etc.

Again a modified t-test was used to compare the standardized band-energies per animal exposed to GB with the averaged standardized band energies at the corresponding time intervals from the six air-exposed animals (controls). Animals provided with Alzet pumps containing saline and exposed to air for 5 h were compared with similarly pretreated animals exposed to different concentrations of GB for 5 h. Animals provided with Alzet pumps containing pyridostigmine bromide and exposed to air for 5 h were compared with similarly pretreated animals exposed to GB for 5 h.

RESULTS
GB GENERATION, EXPOSURE, AND ANALYSIS

Exposure to GB vapor at the ppt level.
Long term low level exposure studies to GB in the concentration range of 0.2 to 100 $\mu g/m^3$ require sensitive techniques for analysis and control of the vapor concentrations. Therefore a new configuration was developed, involving generation of the vapor, the construction of the exposure chamber and the appropriate analysis system.
The volatility of GB at 20 °C is ca. 15,000 mg/m^3 [NATO Handbook for Sampling & Identification of Chemical Warfare Agents, Vol III, edition 3, 1988]. In order to obtain the desired concentration of GB vapor, cooling of the agent storage vessel to ca. 5 °C by means of a thermostated bath as well as three dilution steps (ranging from 1 to 50 till and from 1 to 100) were needed. Also attention had to be paid to prevent pressure build-up and cold spots and the occurrence of chemically active sites. All these factors influence the fluctuation of the vapor concentration. Where possible glass tubing was used which was thermostated either by thermostated tubing or by packing with thermostated material. A total of five pressure ventils were used not only for safety reasons but also for allowing a constant pressure gradient in the system (two of these were used in the dilution steps, see also Fig 8), thus allowing a constant vaporization. The other three pressure ventils were used in combination with manometers in the nitrogen and air supply and on the exposure chamber.

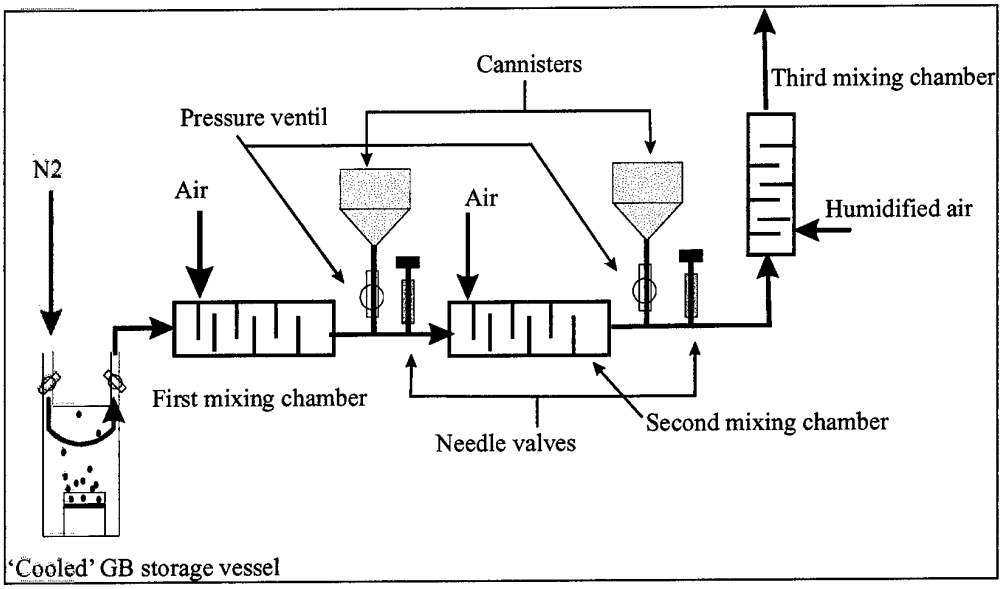

Fig 8. Schematic representation of the dilution steps for generation of a low vapor concentration of sarin in air.

The linearity in the change of the vapor concentration with the 'turns' of the needle valve is influenced by active sites and diffusion effects in a uncontrollable way, i.e., a sudden concentration drop or a longlasting high concentration can occur. Also memory effects due to diffusion are more pronounced, especially if the flow through the needle valve is rather low. By using more than two or three dilution steps the 'agent-flow' through the system can be increased, thereby minimizing the memory or diffusion effects.

The guinea pig or marmoset can be placed inside the exposure chamber, see Fig 9. By opening the valves I to IV, the animals are exposed to GB vapor. By using a carbon filter and a vacuum pump the vapor is let out and cleaned. During the handling of the animals (i.e., placing in or out) the vapor is by-passed by switching the valves *I* to *IV*. During this

switching the chamber is flushed with clean air. During the exposure the vapor is sampled just in between the valve *II* and the exposure chamber and directed to the GC for analysis. The pressure in the chamber is kept at 0.5 kPa.

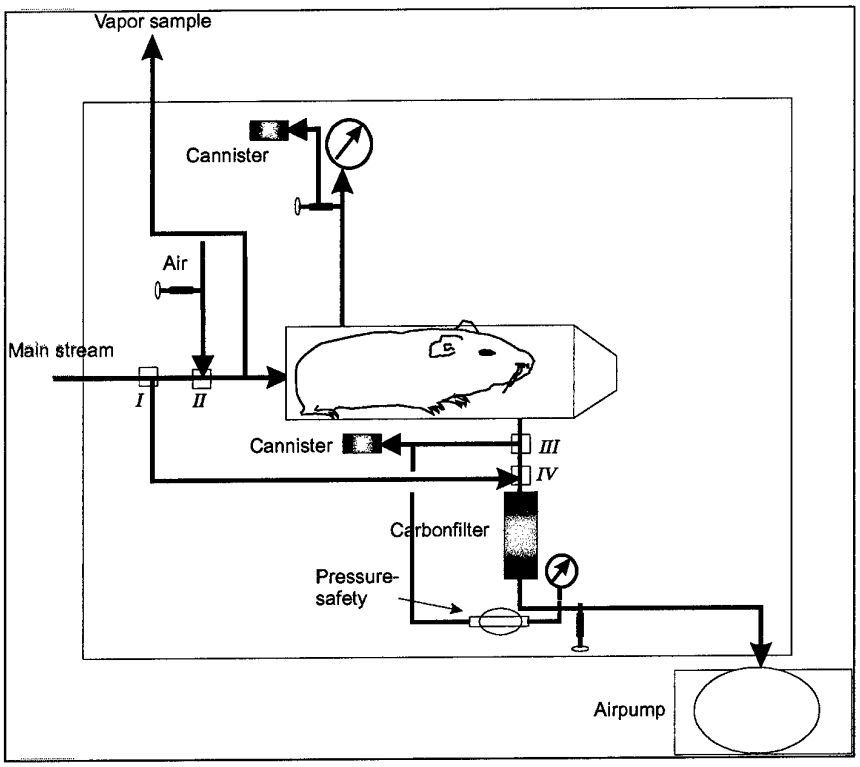

Fig 9. Schematic representation of the exposure chamber. I to IV are 3-way valves, used for by-passing and introducing the vapor. A small fan is used for mixing the vapor at the back of the guinea pig. A combination of manometers, needle valves and pressure ventils is used for controlling the pressure inside the exposure chamber, and for safety.

A number of techniques are available for vapor sampling and analysis. A fixed volume gas sampling valve in combination with gas chromatographic analysis is in most cases satisfactory. However, the minimum detectable concentration with such a system is ca. 0.1-1.0 mg/m^3 when using an NP detector. In order to realize an absolute detection limit of approximately 1 pg of GB unrealistically large injection volumes should be used. Off-line sampling methods, e.g., with a solid or liquid adsorbent allows a preconcentration of a larger sample volume. However, on line detection is preferred because the proces can be controlled more adequately, with the time period between analysis and action (e.g., flow adjustment) as short as possible. A new analytical configuration was constructed in which vapor samples were concentrated in a cold trap, followed by flash heating and analysis with GC-NPD. In this way sarin levels $\geq$ 0.1 µg/m^3 could be analyzed semi-continuously with 2-5 min time intervals.

The gas chromatograph was equipped with a nitrogen phosphorus detector (NPD), an on-column injector and an externally controlled sampling device. This device was constructed in house by using a Valco (Schenkon, Switzerland) 6-port injection valve with an electronic actuator, the cold trap and Music communicator of the 'MUSIC-system' (Chrompack, Middelburg, Netherlands) and in house constructed hardware boxes, for data communication.

A combination of uncoated deactivated fused silica tubing and a very short column were used to perform gas analysis of GB and calibration of the GC on the same detector. A DB1 column (length: 10 m; i.d.: 0.32 mm, film thickness: 1 µm) was installed onto the on-column injector and connected to an all glas Y-press fit connector (see Fig 10: grey spot in the GC). The gas sampling valve was connected to the other 'leg'of this Y-press fit with a piece of uncoated deactivated fused silica (length: ca 40 cm; i.d.: 0.32 mm). Finally, 20 cm of a DB1 column (i.d.: 0.32 mm, film thicknes: 1 µm) was used to connect the Y-press fit to the detector.

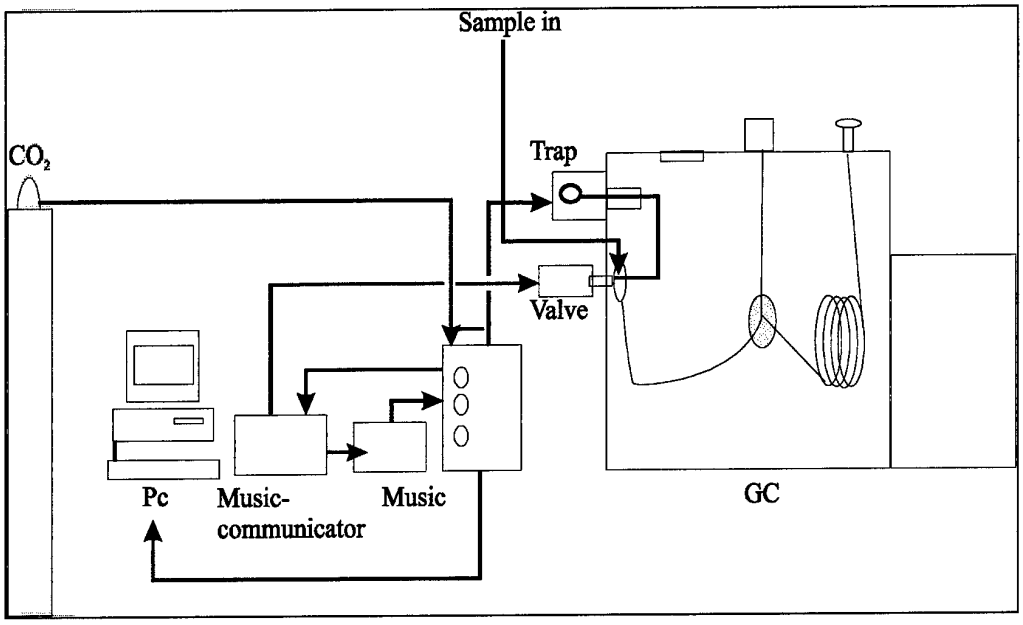

Fig 10. Schematic representation of the configuration for low level vapor analysis. The grey spot in the GC is the all glass Y-press fit connector. Red line represents the electronic control signals from the Music comm. to the sampling valve. Blue lines represent the flow of CO_2 for cooling. Other arrows represent the electronic back coupling to the MUSIC communicator and PC.

The generated vapor flow through the exposure chamber is ca. 5 L/min. Just before the entrance of the exposure chamber, a vapor sample was taken from the main stream by means of a piece of PEEK tubing and directed through a 6-port Valco sampling valve (see also Fig 10 and Fig 11). A vapor sample was concentrated in the cold trap for ca. 0.5 min (depending on required volume). The further analysis took 2.5 min. Consequently every 2-5 min a vapor sample can be analyzed. Connected to this valve, a piece of uncoated fused silica (i.d.: 0.32 mm length: ca. 60 cm) is led through the metal cold trap of the MUSIC apparatus and guided back ('Cold Loop Trap'). The flow in the cold trap (-70 °C) is kept constant at 20 ml/min during the sampling period by means of a needle valve and pump.
The sample is concentrated in the fused silica trap to a very small volume (<10 µl). The sample volume can be regulated by either the flow or the sampling time.

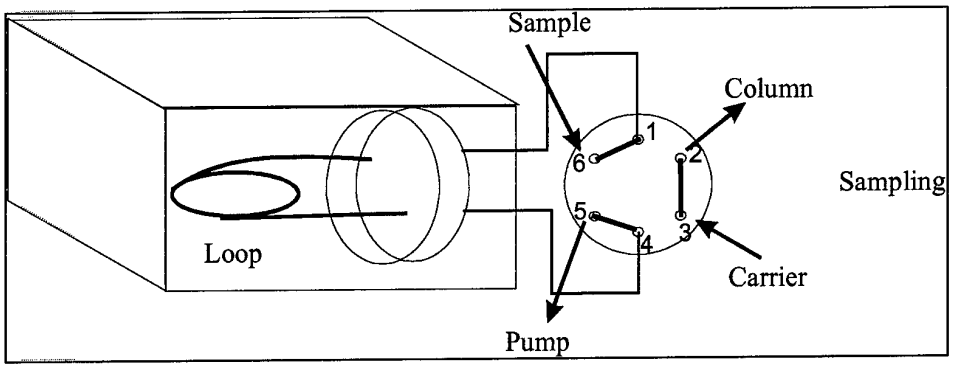

Fig 11. Schematic representation of the 'Cold Loop Trap'.

After the trapping period the valve is switched and the trap is heated rapidly to 120 °C. The trapped components are injected into the connected column and finally detected with the NPD. Fig 12 shows a typical chromatogram of the vapor analysis.
The GC was calibrated by injecting 1 µl of standard solutions, containing sarin in ethyl acetate in concentrations varying from 0.1 to 10 ng/ml.

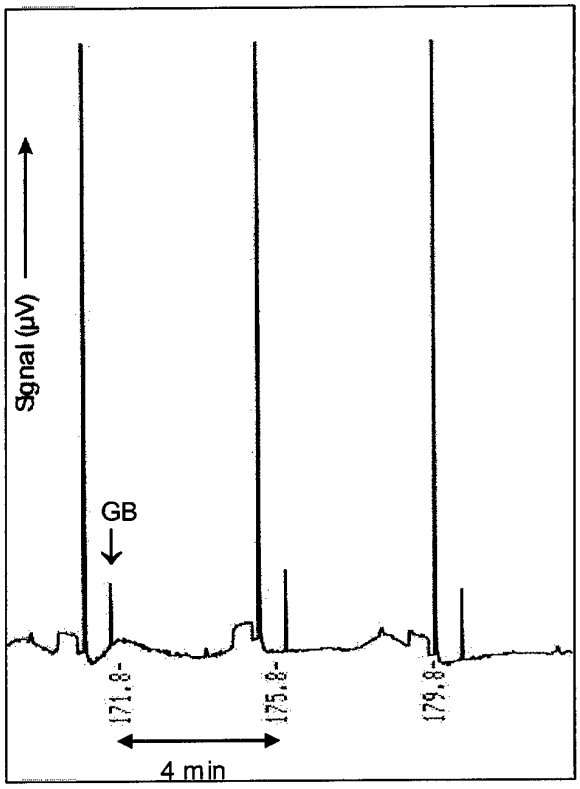

Fig 12. Chromatogram of a 'real' vapor sample. The concentration of sarin was approx. 0.1 µg/m³ (ca. 4.5 ppt).

In order to validate both techniques, the fixed volume gas sample loop and the 'Cold Sample Loop', the effectiveness of both gas sampling techniques was investigated. For the validation of the more traditional fixed volume gas sample loop, a technique was used in which the sample flow was passed through a liquid adsorbent (ethyl acetate) for a fixed period of time and a fixed sample flow. Subsequently, the liquid-trapped agent (1 µl) was analyzed by means of gas chromatography with NP detection.

This method of sarin vapor trapping in ethyl acetate was validated by using a standard solution of GB in ethyl acetate (ca. 33 ng/ml) and passing through for a period of time (40 min) a stream of air (25 ml/min). Subsequently, the remaining GB in ethyl acetate was quantified based on a separate standard solution of GB in ethyl acetate. In this way it was found that 92 ± 3% of the GB had remained in the solution. By using this technique vapor concentrations of 100 and 50 $\mu g/m^3$ were analyzed and the results considered as a validation (see Table 1). Liquid adsorption data given in this table were corrected for trapping efficiency. These vapor concentrations sampled with this verifying technique could be analyzed with an efficiency of 90-95% and 80-85 %, respectively.

Table 1. Validation of the cold trapping vapor analysis technique at intermediate low levels.

Vapor concentration	Cold trapping on-line analysis	Liquid adsorption	Efficiency
100 $\mu g/m^3$	91-96 $\mu g/m^3$	101 $\mu g/m^3$	90-95 %
50 $\mu g/m^3$	38-41 $\mu g/m^3$	48 $\mu g/m^3$	80-85 %

The limit of the sensitivity of the verification method as described above was at ca. 50 $\mu g/m^3$ of GB vapor. In order to evaluate the effectiveness of the cold trapping technique at the lower sarin vapor levels a different and independent sampling technique was used. The PEEK tubing which led to the sampling valve was connected to a TCT (Thermal Cold Trap, Chrompack, Middelburg, Netherlands) sampling tube filled with Tenax T.A. (60-80 mesh, ca 150 mg). Next, during a fixed period of time and using a fixed sample flow the vapor is trapped on this Tenax. Subsequently, the tube is disconnected and capped. Next, the tube is transferred to a Carlo Erba Gas Chromatograph ecquipped with a TCT injector, a two dimensional column switching system, MUSIC (Chrompack, Middelburg, Netherlands), FID and NP detection. This system was extensively described in the Experimental Part of this final report and was earlier published by Polhuijs et al (1997).

First the efficiency of trapping onto the Tenax (validation) was investigated by injecting 10 µl of a stock solution of sarin in ethyl acetate (103 ng/ml) on the Tenax. Next a stream of nitrogen of 250 ml/min was passed through the Tenax for increasing periods of time before analyzing with the latter gas chromatographic system. The results of the trapping and desorbing efficiency are given in Fig 13.

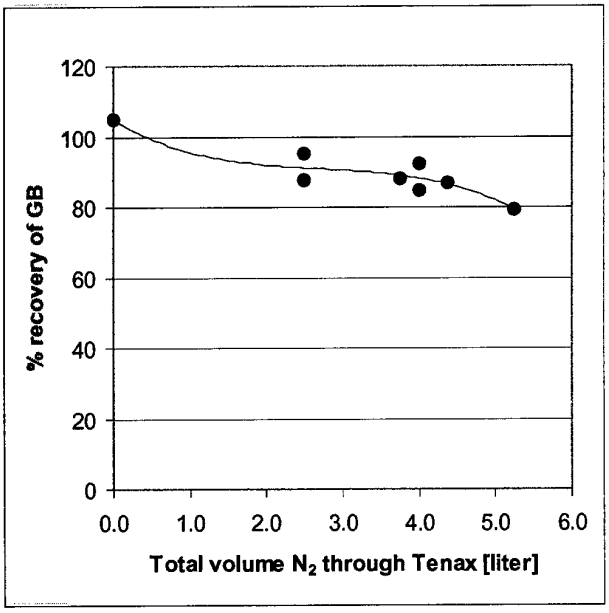

Fig 13. Trapping and desorption efficiency of sarin on ca. 100 mg of Tenax TA charged with 10 µl of a stock solution of sarin in ethyl acetate (103 ng/ml) followed by passing through a stream of nitrogen at 250 ml/min for increasing periods of time and subsequent analysis with the TCT-MUSIC-NPD analytical configuration.

From the results shown in Fig 13 it can be concluded that by limiting the total sample volume to ≤ 1 L of vapor (i.e., 10 min with a flow of 100 ml/min) the overall efficiency is better than 95 %.

Low level (ca. 0.1 µg/m^3) GB vapor concentration was generated and analyzed with the 'Cold Sample Loop'. See Fig 14 for a schematic representation of the procedure. Numbers between brackets refer to sampling numbers in Fig 14. After five analyses (1) the sample tube was disconnected from the GC valve and connected to the first (2) of two Tenax sample tubes. The sample was passed through the Tenax for 10 min at a flow of 100 ml/min. Next, the tubing was disconnected and connected to the GC valve for another set of analysis (3). Once again the tubing was disconnected from the gas valve and connected to the second Tenax sample tube (4) and the vapor was charged on this tube. Finally, the tubing was disconnected from the tube and connected to the gas valve for the last set of analysis (5). The GB vapor concentration for the 'Cold Sample Trap' was calculated by averaging the individual vapor concentrations in each of the time slots 1, 3, and 5.

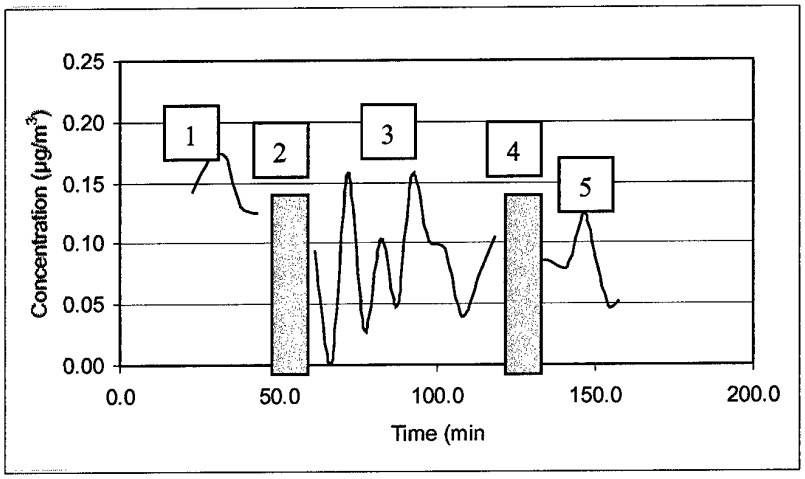

Fig 14. Time course of the vapor concentration determined with the cold trapping technique (1) before the first Tenax sampling, (2) the 10 min sample period for the first Tenax/ TCT analysis, (3) in between the two Tenax/ TCT analysis, (4) the 10 min sample period of second Tenax/ TCT analysis and (5) after the second Tenax sampling.

Results of the measurements as indicated in Fig 14 are given in Table 2. The difference between the off-line analysis with the TCT-MUSIC configuration and the on-line 'cold-trapping' sampling technique is approximately 10 % at these low vapor levels of ca. 0.1 µg/m^3 sarin in air.

Table 2. Validation of the cold trapping vapor analysis technique at low level.

TCT sampling			TCT analysis	Cold trapping on-line analysis	Sampling number
Flow (ml/min)	Time (min)	Volume (L)	Concentration ($\mu g/m^3$)		
				0.15	1
100	10	1.0	0.09		2
				0.08	3
100	10	1.0	0.13		4
				0.08	5
Averaged			0.11	0.10	

It is concluded that this method of cold trapping and flash heating is a reliable way in routine measurements of low level vapors of sarin in air at the low ppt level.
In theory an unlimited volume of vapor can be concentrated into the cold trap and subsequently analyzed. In practice, due to contamination and humidity of the vapor, the maximum sample size was 15-20 ml. This was sufficient for the purpose of these investigations.

GUINEA PIG EXPERIMENTS

The Lowest Observable Effect Level (LOEL) of GB exposure for vehicle-pretreated guinea pigs

As described in Materials & Methods, 6 vehicle-pretreated (Gp13-Gp18) were used to determine the *LOEL*. One animal per day was exposed to 0.05 – 0.6 $\mu g/m^3$ GB for a period of 5 h. During exposure blood samples (500 – 700 µl) were taken every 30 min for internal dose assessment.

One exposure experiment (Gp16) had to be terminated because the required GB vapor concentration was not generated in time after starting the exposure. The generated GB vapor concentrations during the separate exposures of the individual vehicle-pretreated animals are given in Fig 15 (panels Gp13-Gp18). In most cases the generation of GB was fairly constant. The mean GB vapor concentration generated, varied from 0.05 – 0.6 $\mu g/m^3$ (compare panel Gp18 and Gp14). Consequently, the exposure period needed to detect the GB internal dose in blood samples, varied between the animals from 30 – 120 min. Apparently, during exposure to a low concentration it took more time before GB could be detected in blood samples if compared with exposure to a high concentration. The vertical bars indicate the first time points at which the internal doses could be detected in a reliable way. The GB analysis was considered to be reliable if the signal-to-noise ratio was equal or greater than 2 ($S/N \geq 2$). The shaded cells in Table 3 contain the first internal doses in the course of the exposures which could be detected reliably. The mean individual exposure concentrations of GB ($\mu g/m^3$) generated over the period of time between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected reliably (until vertical bar) are shown in Table 4. Time-based average of the vapor concentrations measured every 2-5 min, was used to calculate the mean concentration over a particular period of exposure time. The individual *LOEL* levels of exposure were calculated on the basis of these values and were in the range of 0.004 – 0.017 $mg.min.m^{-3}$. The averaged *LOEL* level for vehicle-pretreated guinea pigs (Gps 13 – 18) was calculated to be 0.010 ± 0.002 $mg.min.m^{-3}$ (mean ± SEM, n = 5) (Table 15).

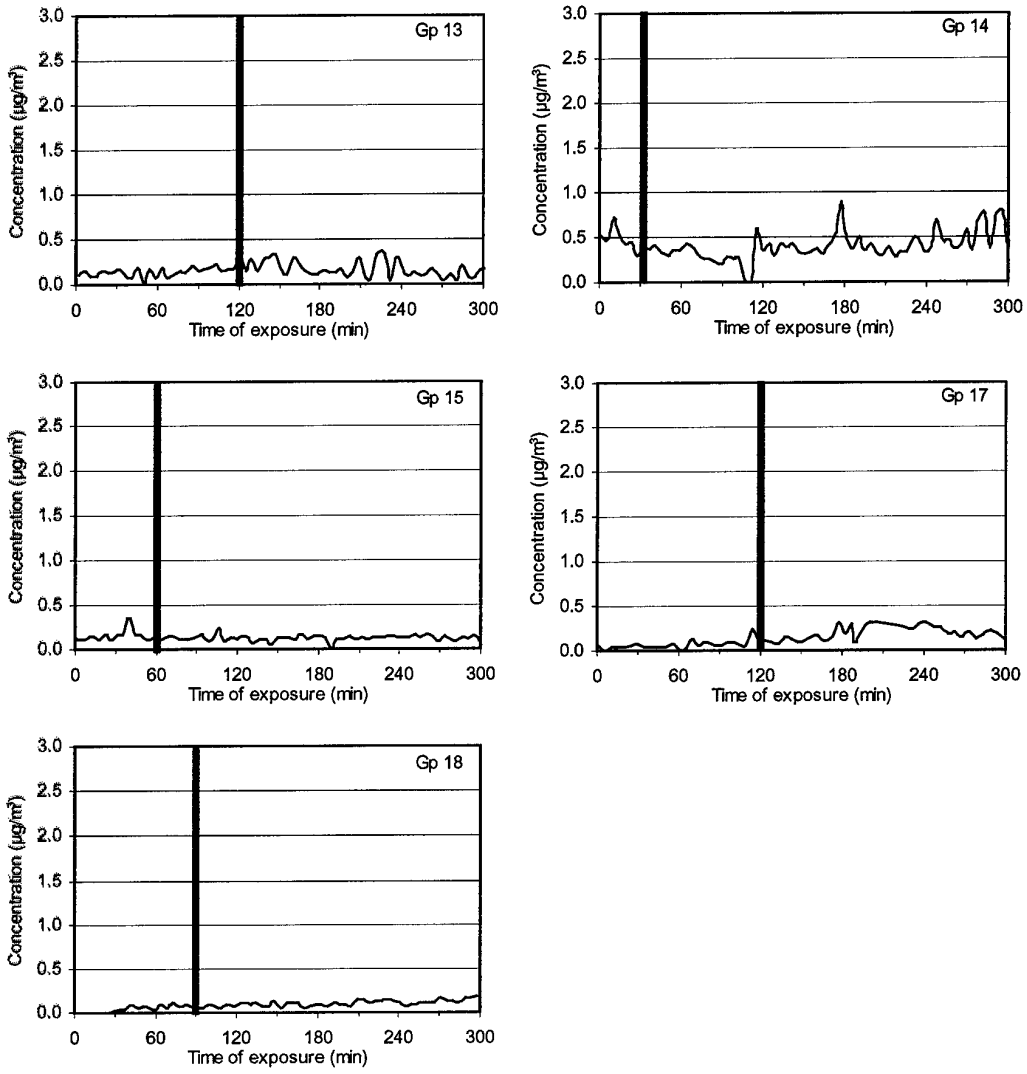

Fig 15. Time course (horizontal axis) of generated GB vapor concentrations (vertical axis) in air during exposure of vehicle-pretreated animals. The vertical bars indicate the first time points at which the internal doses could reliably (S/N ≥ 2) be detected.

Table 3. Fluoride-regenerated GB concentrations (pg/ml) determined in approximately 0.5 ml blood samples drawn from vehicle-pretreated guinea pigs (Gp 13-18) exposed to 0.05 – 0.6 µg/m³ GB vapor in air. The shaded cells contain the first internal doses during the exposures which could be detected in a reliable (S/N ≥ 2) way. Gp numbers correspond to those shown in Fig 15. Note that experiment Gp16 was terminated because the required GB concentration could not be generated in time. N.a. = not analyzed; b.d. = below detection limit (S/N < 2).

Time [min]	Gp13	Gp14	Gp15	Gp16	Gp17	Gp18
0	b.d.	b.d.	b.d.		b.d.	b.d.
30	b.d.	21.3	b.d.		b.d.	b.d.
60	b.d.	32.1	5.6		b.d.	b.d.
90	b.d.	n.a.	6.5		b.d	3.4
120	11.3	n.a.	7.2		2.0	b.d.
150	9.5	n.a.	n.a.		2.2	5.1
180	17.3	66.6	n.a.		3.9	n.a.
210	13.4	n.a.	n.a.		n.a.	n.a.
240	16.9	n.a.	n.a.		5.5	n.a.
270	18.0	n.a.	n.a.		n.a.	7.3
300	16.4	102.4	14.0		7.1	n.a.

Table 4. Mean exposure concentration of GB vapor in air (µg/m³) generated over the period of time (LOEL-time) between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected in a reliable (S/N ≥ 2) way, and calculation of the individual LOEL. Gp numbers correspond to those shown in Fig 15 and Table 3.

Animal	Gp13	Gp14	Gp15	Gp17	Gp18
Time to *LOEL* [min]	120	30	60	120	90
Mean (± sem)* GB vapor conc. to time to *LOEL* [µg/m³]	0.14 ± 0.01	0.47 ± 0.05	0.15 ± 0.02	0.06 ± 0.01	0.04 ± 0.01
LOEL [mg.min.m⁻³]	0.017	0.014	0.009	0.008	0.004
Mean (± sem) *LOEL* [mg.min.m⁻³]	0.010 ± 0.002				

** Time-based average of vapor concentrations measured at 2-5 min intervals*

The Lowest Observable Effect Level (LOEL) of GB exposure for pyridostigmine-pretreated guinea pigs

The pyridostigmine-pretreated animals were numbered Gp19 – Gp24 (Fig 16). The inter-individual differences in generated GB concentrations were in the range between 0.05 – 0.4 µg/m³ (see panel Gp19 – Gp24). The exposure period needed to detect the GB internal doses in blood samples varried between the individual animals from 60 – 180 min depending on the level of exposure. The shaded cells in Table 5 contain the first internal doses in the time course of the individual exposures which could be detected reliably (S/N ≥ 2). The mean individual exposure concentrations of GB (µg/m³) generated over the period of time between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected reliably (S/N ≥ 2) (until vertical red bar in Fig 16) are shown in Table 6. The individual *LOEL* levels were calculated on the basis of these values and were in the range between 0.0027 – 0.0200 mg.min.m⁻³. The averaged *LOEL* level for pyridostigmine-

pretreated guinea-pigs was calculated to be 0.014 ± 0.003 mg.min.m^{-3} (mean ± SEM, n = 6). This was statistically not significantly different ($p > 0.05$) from that of vehicle-pretreated animals.

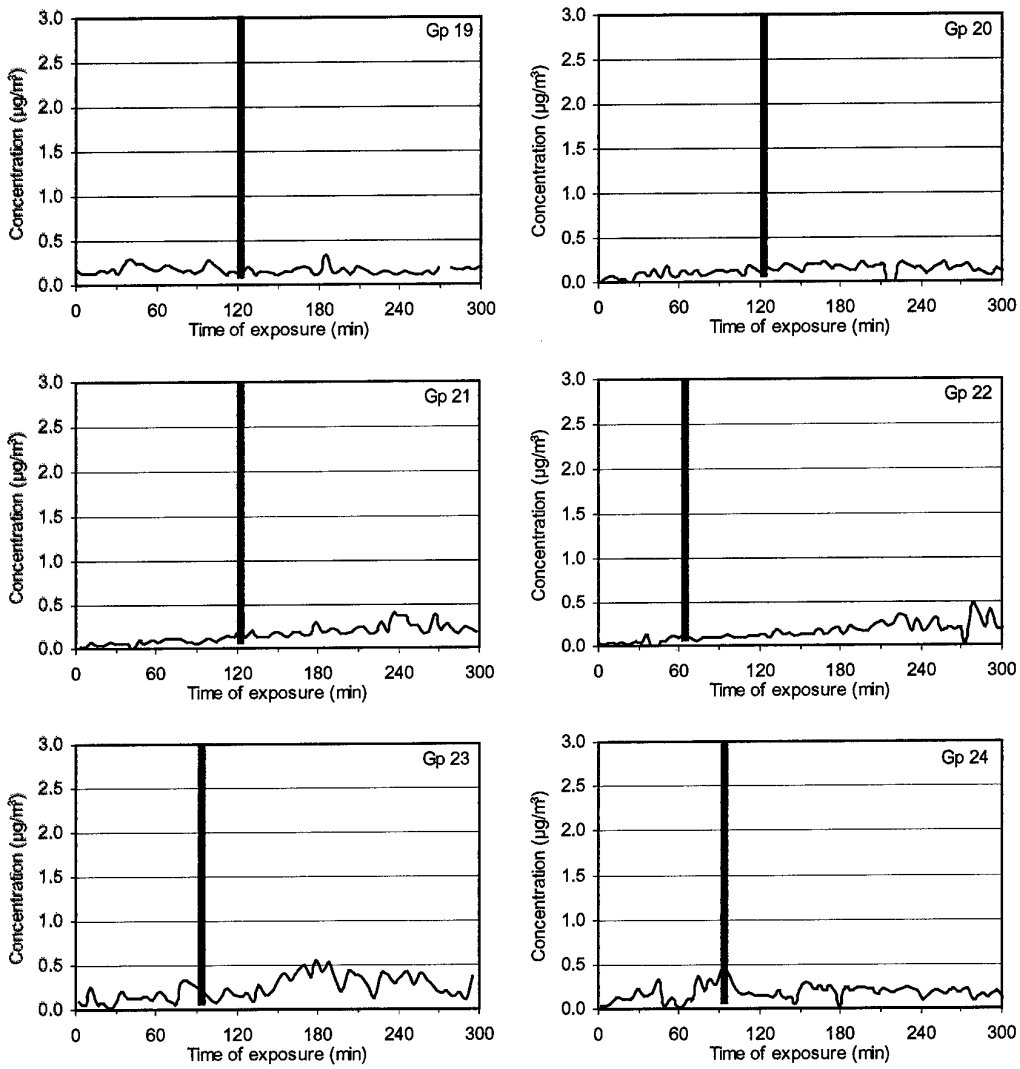

Fig 16. Time course (horizontal axis) of generated GB vapor concentrations(vertical axis) in air during exposure of pyridostigmine-pretreated animals. The vertical red bars indicate the first time point at which the internal doses could reliably (S/N ≥ 2) be detected. The concentration of GB was measured semi-continuously at 2-5 min intervals.

Table 5. Fluoride-regenerated GB concentrations (pg/ml) determined in 0.5 ml blood samples drawn from pyridostigmine-pretreated guinea pigs (Gp 19-24) exposed to 0.05 – 0.4 µg/m³ GB vapor in air. The shaded cells contain the first internal doses during the exposures which could be detected in a reliable (S/N > 2) way. Gp numbers correspond to those shown in Fig 16. N.a. = not analyzed; b.d. = below detection limit (S/N < 2).

Time [min]	Gp19	Gp20	Gp21	Gp22	Gp23	Gp24
0	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
30	b.d.	b.d	b.d	b.d.	b.d.	b.d
60	b.d.	b.d	b.d	1.2	b.d.	b.d.
90	b.d.	b.d	b.d	2.0	6.3	2.8
120	4.7	6.5	6.3	3.8	8.8	4.7
150	6.5	2.7	1.2	4.6	n.a.	5.4
180	n.a.	4.4	2.2	4.9	19.3	n.a.
210	9.2	n.a.	2.7	n.a.	n.a.	n.a.
240	n.a.	n.a.	3.8	8.9	22.1	9.2
270	n.a.	7.1	n.a.	n.a.	n.a.	n.a.
300	10.8	n.a.	5.6	11.3	26.2	14.8

Table 6. Mean exposure concentration of GB vapor in air (µg/m³) generated over the period of time (LOEL-time) between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected in a reliable (S/N ≥ 2) way in pyridostigmine-pretreated animals, and calculation of the individual LOEL. Gp numbers correspond to those shown in Fig 16 and Table 5.

Animal number	GP19	GP20	GP21	GP22	GP23	GP24
Time to *LOEL* [min]	120	120	120	60	90	90
Mean (± sem)* GB vapor conc. to time to *LOEL* [µg/m³]	0.18 ± 0.01	0.09 ± 0.01	0.08 ± 0.03	0.04 ± 0.02	0.20 ± 0.05	0.17 ± 0.03
LOEL [mg.min.m^{-3}]	0.022	0.011	0.010	0.002	0.018	0.015
Mean (± sem) *LOEL* [mg.min.m^{-3}]	0.014 ± 0.003					

** Time-based average of vapor concentrations measured at 2-5 min intervals.*

The Lowest Observable Adverse Effect Levels (LOAEL) of GB exposure for guinea pigs

Vehicle-pretreatment

The GB vapor concentrations generated to determine the *LOAEL* levels (C.t) of exposure at which significant ($p < 0.05$) changes were being expected to emerge regarding pupil size, EEG, VER, startle-response, and shuttle-box behavior of vehicle-pretreated guinea pigs, are shown in Fig 17. The aim was to expose animals for 5 h exposure periods to the following concentrations of GB: 150, 50, 25, 15 and 7.5 $\mu g/m^3$, two animals per concentration. One of these two animals was used for taking blood samples, the other one for measuring miosis, EEG/VER during exposure, and for performance-testing (startle-response, shuttle-box) at the end of the 5 h exposure period.

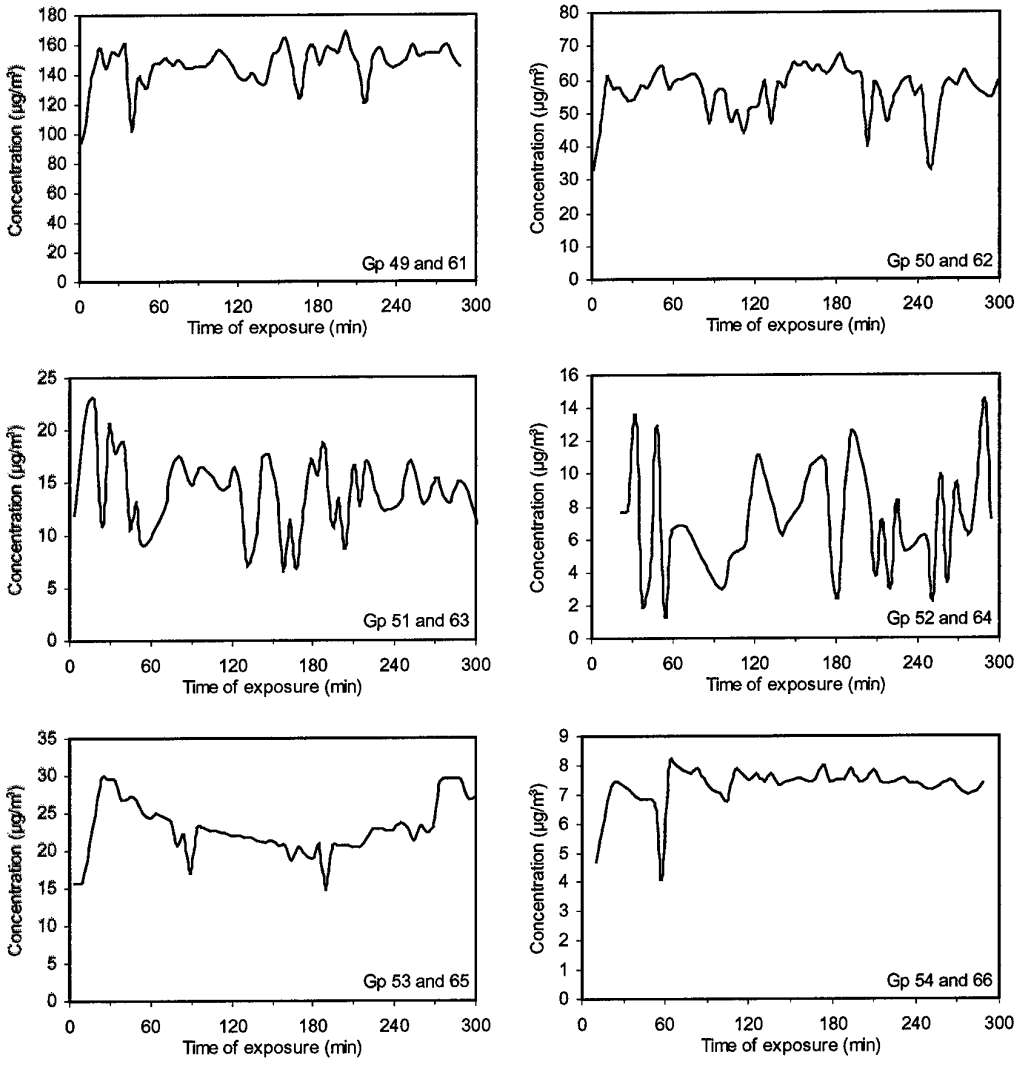

Fig. 17. Time course (horizontal axis) of generated GB vapor concentrations($\mu g/m^3$) in air (vertical axis) during exposure of vehicle-pretreated animals for determination of LOAEL levels. The concentration of GB was measured semi-continuously at 2-5 min intervals.

Two experiments were performed in which two animals were exposed to approximately 7.5 $\mu g/m^3$: Gp52 + 64 were exposed to 7.1 $\mu g/m^3$, Gp54 + 66 to 7.4 $\mu g/m^3$. The calculated time-base averaged concentrations that were actually achieved are given in Table 7.

Table 7. Calculated actual mean concentrations of GB vapor to which vehicle-pretreated guinea pigs were exposed in order to determine the LOAEL (C.t) levels of exposure.

Guinea pig no.	Mean (± sem)* GB concentrations ($\mu g/m^3$) between t = 0 and t = 300 min
Gp49 + 61	146.2 ± 1.9
Gp50 + 62	56.4 ± 1.0
Gp51 + 63	14.3 ± 0.5
Gp52 + 64	7.1 ± 0.5
Gp53 + 65	22.9 ± 0.5
Gp54 + 66	7.3 ± 0.1

** Time-based average of vapor concentrations measured at 2-5 min intervals.*

Pyridostigmine-pretreatment

The GB vapor concentrations generated to determine the *LOAEL* levels (C.t) of exposure at which significant ($p < 0.05$) changes are expected to emerge regarding pupil size, EEG, VER, startle-response, and shuttle-box behavior of pyrodostigmine-pretreated guinea pigs, are shown in Fig 18. The aim was to expose animals for 5 h exposure periods to the following concentrations of GB: 200, 150, 50, 25, 15 and 7.5 µg/ m^3, two animals per concentration. One of the two animals was used for taking blood samples, the other one for measuring miosis, EEG/VER during exposure, and for performance-testing (startle-response, shuttle-box) at the end of the 5 h exposure period. The calculated mean concentrations that were actually achieved are given in Table 8.

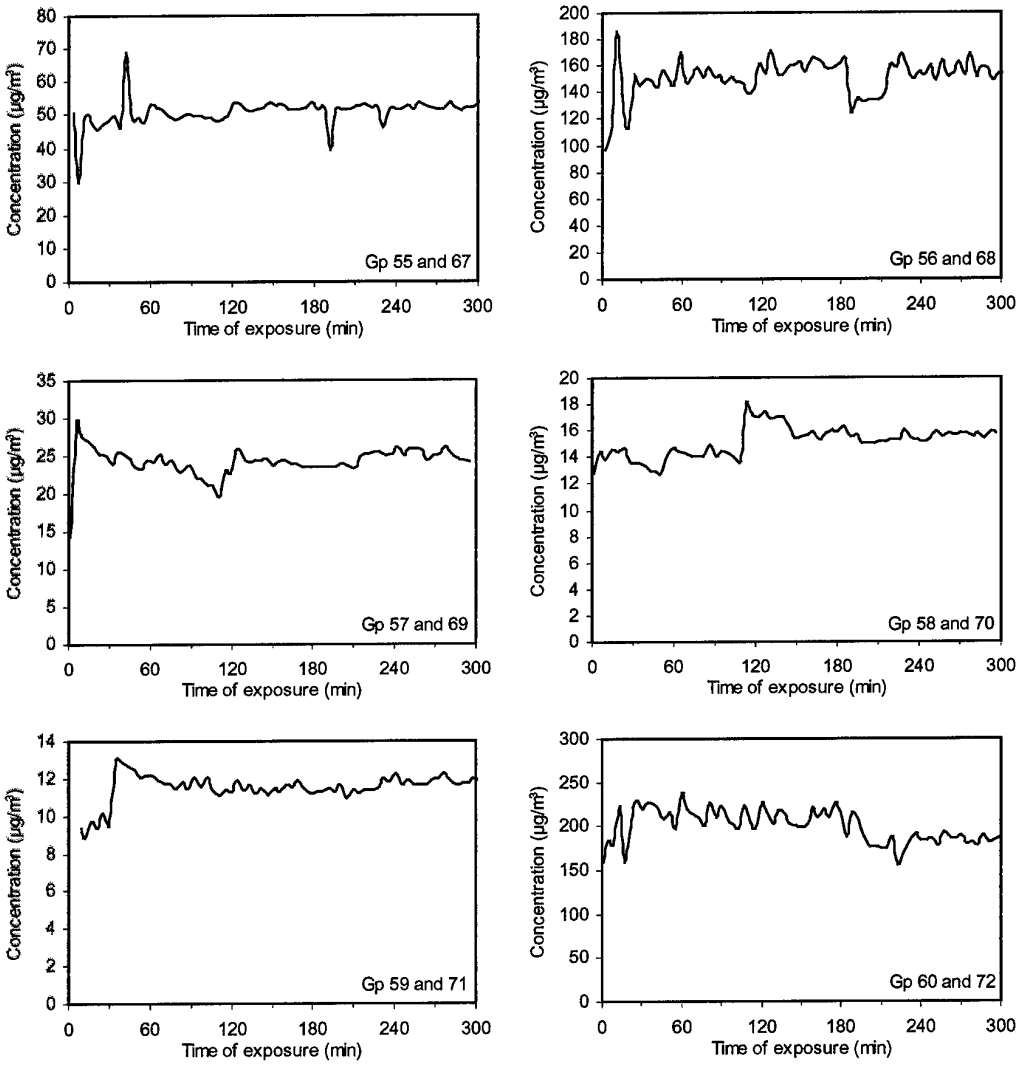

Fig. 18. Time course (horizontal axis) of generated GB vapor concentrations(µg/m^3) in air (vertical axis) during exposure of pyridostigmine-pretreated animals for determination of LOAEL levels. The concentration of GB was measured semi-continuously at 2-5 min intervals.

Table 8. Calculated actual mean concentrations of GB vapor to which pyridostigmine-pretreated guinea pigs were exposed in order to determine the LOAEL (C.t) levels of exposure.

Guinea pig no.	Mean (± sem)* GB concentrations ($\mu g/m^3$) between t = 0 and t = 300 min
Gp55 + 67	50.8 ± 0.5
Gp56 + 68	151.3 ± 1.2
Gp57 + 69	24.2 ± 0.2
Gp58 + 70	15.2 ± 0.1
Gp59 + 71	11.5 ± 0.1
Gp60 + 72	199.5 ± 2.3

** Time-based average of vapor concentrations measured at 2-5 min intervals.*

1. Miosis

Vehicle-pretreatment

A 5 h whole-body exposure of vehicle-pretreated guinea pigs to GB vapor concentrations in the range of 7.5 – 150 $\mu g/m^3$ resulted in significant ($p < 0.05$) and concentration-related decreases in pupil size (miosis) compared to the averaged pupil size (0.77) in naïve animals ($n = 6$) at the end of a 5-h exposure to air (see Fig 19). A decrease of 5% in pupil size was significantly ($p < 0.05$) different from control value.

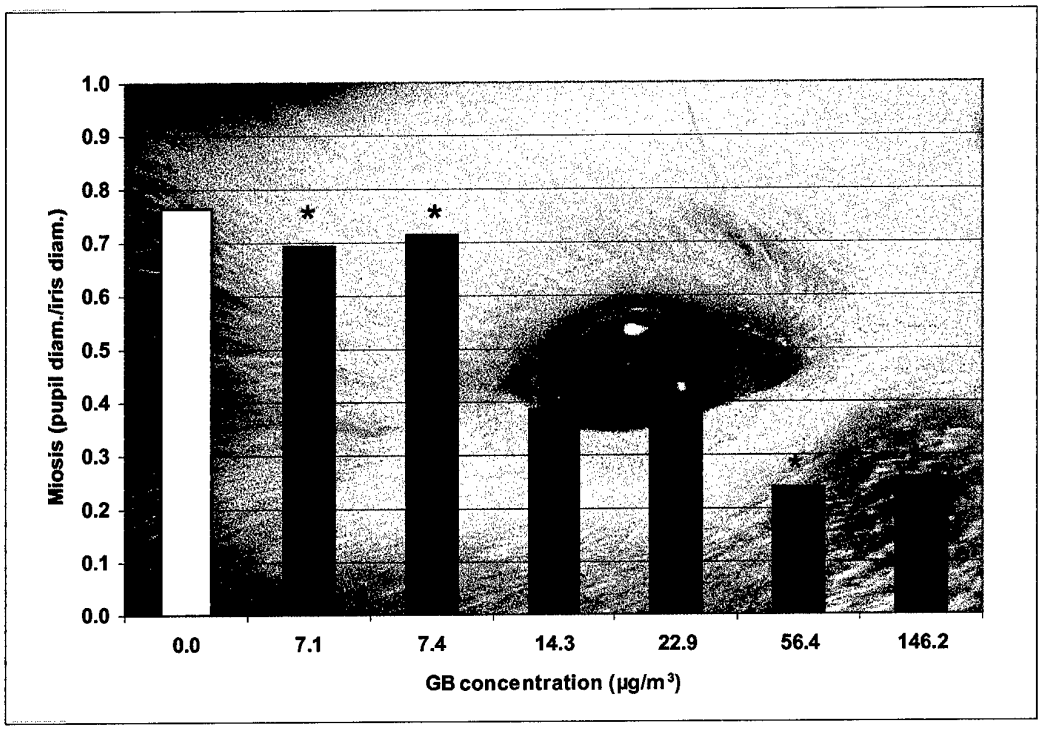

*Fig 19. Miosis of restrained conscious guinea pigs provided with Alzet pumps containing vehicle, after a 5 h exposure to either air (n=6) (mean ± SEM) or various mean concentrations of GB (7.1, 7.4, 14.3, 22.9, 56.4 or 146.2 $\mu g/m^3$, one animal per concentration). *, significantly different from the mean value of the air-exposed animals, $p < 0.05$.*

In Table 9 the exposure times needed to achieve significant ($p < 0.05$) miosis during exposure to the various mean GB concentrations, and the corresponding C.t values are given.

Table 9. Exposure times needed to achieve significant ($p < 0.05$) miosis during exposure of vehicle-pretreated guinea pigs to the various mean GB concentrations, and the corresponding C.t values. Nm ≡ not measured.

Vehicle-pretreatment		
Mean (± sem) conc.of GB exposure ($\mu g/m^3$)	Time (min) to significant miosis ($p<0.05$)	C.t ($mg.min.m^{-3}$)
-	N.m	-
7.3 ± 0.1	270	1.98
15.5 ± 1.5	67	1.04
24.9 ± 2.4	44	1.10
52 ± 3	38	1.99
127 ± 10	22	2.75
	Mean ± sem	1.8 ± 0.3

The mean C.t value (Table 9) was taken as the *LOAEL* regarding miosis in vehicle-pretreated guinea pigs: 1.8 ± 0.3 $mg.min.m^{-3}$.
At the lowest GB concentration tested (7.3 $\mu g/m^3$) the decrease in pupil size became significant ($p < 0.05$) during the 5th h of exposure, whereas at the higher concentrations (in the range of 15 – 150 $\mu g/m^3$), miosis became manifest much earlier in the 5 h exposure period, see Fig 20 for a typical example.

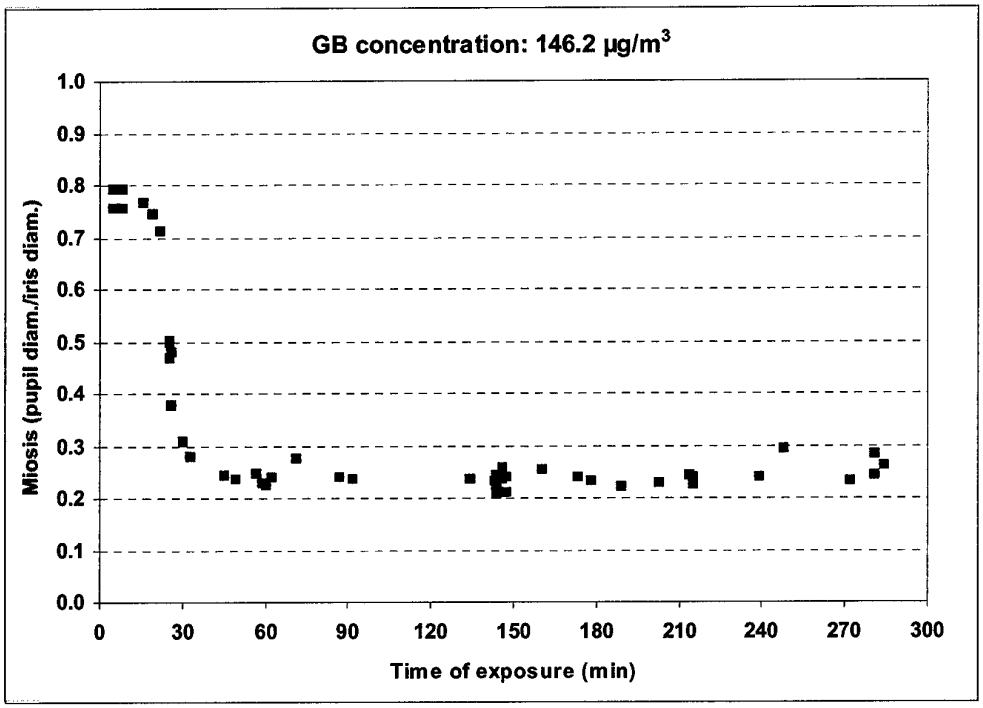

Fig 20. Development of miosis in a vehicle-pretreated guinea pig which were whole-body exposed to an averaged concentration of 146.2$\mu g/m^3$ of GB vapor in air during a 5 h exposure period. Compare Fig 19.

The relationship between the final degree of miosis at the end of a 5 h exposure and the mean GB exposure concentrations was fitted on a linear scale in Fig 21.

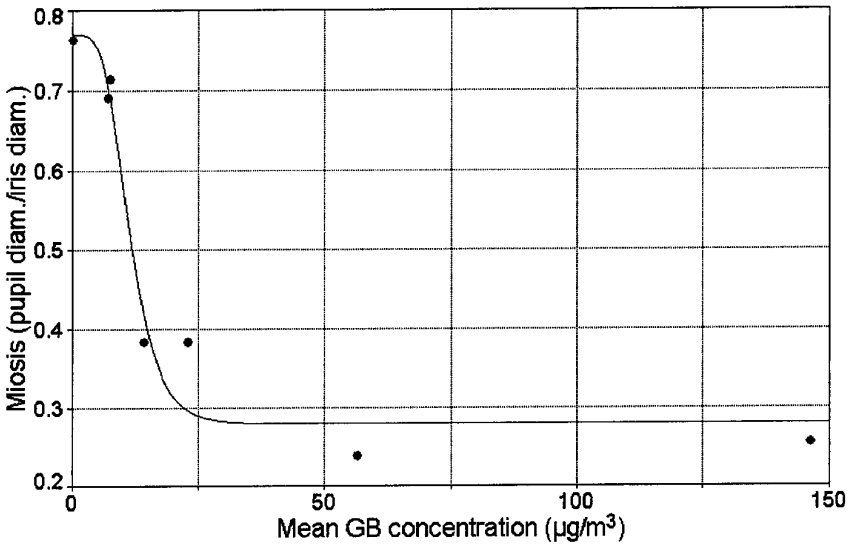

Fig 21. Relationship between the degree of miosis (vertical axis) in vehicle-pretreated guinea pigs at the end of a 5 h exposure to GB, and the mean GB concentrations of exposure (horizontal axis).

Pyridostigmine-pretreatment

A 5 h exposure of pyridostigmine-pretreated animals to various GB vapor concentrations (11.5 – 200 μg/m^3), resulted in significant ($p < 0.05$) and concentration-related decreases in pupil size (miosis) compared to the averaged pupil size (0.77) in naïve animals ($n = 6$) at the end of a 5 h exposure to air (Fig 22). A decrease of 5% in pupil size was significantly ($p < 0.05$) different from control value.

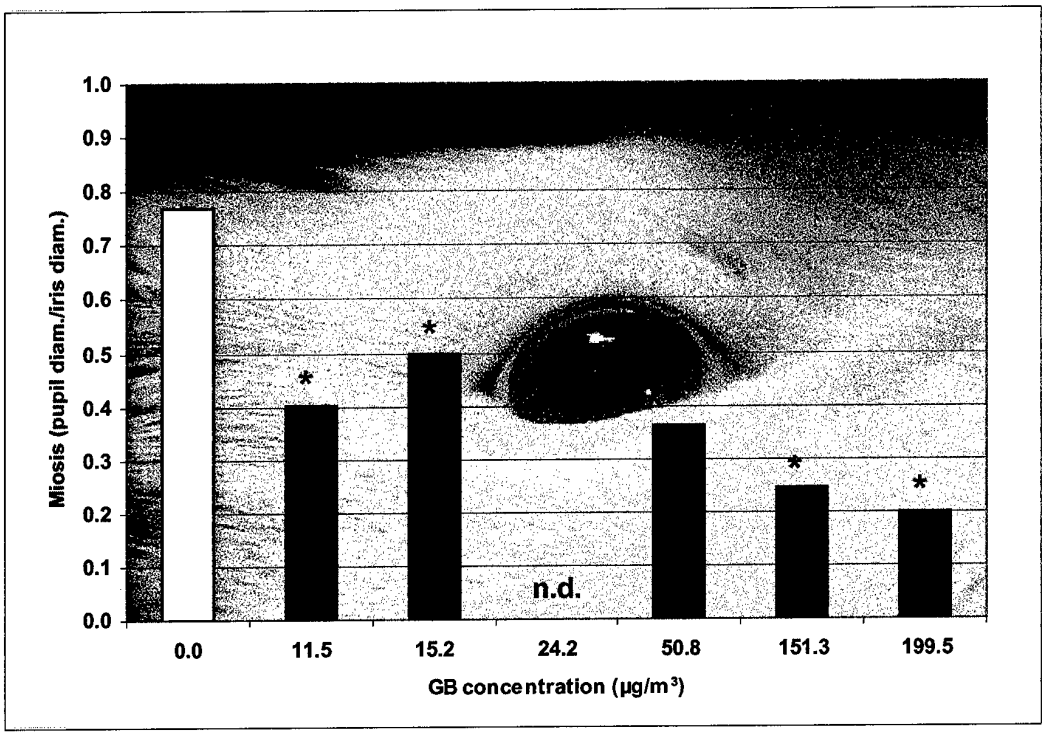

Fig 22. Miosis of restrained conscious guinea pigs provided with Alzet pumps containing pyridostigmine, after a 5 hr exposure to either air (n=6) (mean ± SEM) or various concentrations of GB (11.5, 15.2, 24.2, 50.8, 151.3 or 199.5 μg/m^3, one animal per concentration). Note the significant ($p < 0.05$) concentration-related decreases in pupil size (miosis) during whole-body exposure to GB vapor in air. N.d.: not determined. (, significantly different from the mean value of the air-exposed animals, $p < 0.05$).*

In Table 10 the exposure times needed to achieve significant ($p < 0.05$) miosis during exposure to the various mean GB concentrations, and the corresponding C.t values are given.

Table 10. Exposure times needed to achieve significant ($p < 0.05$) miosis during exposure of pyridostigmine-pretreated guinea pigs to the various mean GB concentrations, and the corresponding C.t values. Nd = not determined.

Pyridostigmine-pretreatment		
Mean (± sem) conc.of GB exposure ($\mu g/m^3$)	Time (min) to significant miosis ($p<0.05$)	C.t ($mg.min.m^{-3}$)
11.3 ± 0.3	85	0.96
14.3 ± 0.2	120.5	1.73
-	Nd	-
43 ± 7	14	0.61
127 ± 15	19.8	2.51
181 ± 12	16.6	3.00
	Mean ± sem	1.8 ± 0.5

The mean C.t value (Table 10) was taken as the *LOAEL* regarding miosis in pyridostigmine-pretreated guinea pigs: 1.8 ± 0.5 $mg.min.m^{-3}$ which is not significantly different from the *LOAEL* calculated for vehicle-pretreated animals.
The degree of miosis at the lowest GB concentration tested (11.3 $\mu g/m^3$), became significant ($p < 0.05$) during the 5th h of exposure. At the higher concentrations (in the range of 15 – 200 $\mu g/m^3$), miosis was manifest much earlier during exposure (not shown). The relationship between the final degree of miosis in pyridostigmine-pretreated animals and the GB exposure concentrations was fitted on a linear scale in Fig 23.

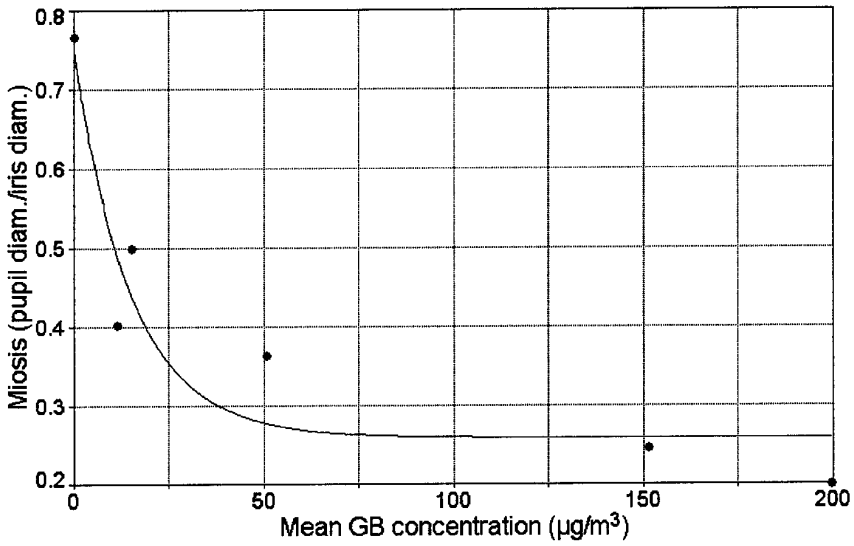

Fig 23. Relationship between the degree of miosis (vertical axis) in pyridostigmine-pretreated at the end of a 5 h exposure to GB, and the mean GB concentrations of exposure (horizontal axis).

Remark: At the BioScience conference at Hunt Valley (2000) a *LOAEL* of 0.2 $mg.min.m^{-3}$ was reported for miosis in pyridostigmine-pretreated guinea pigs. This value was extrapolated from Fig 23 (this report). From that figure the GB concentration (C) was derived at which pupil size would become significantly ($p < 0.05$) different for the first time (at $t = 300$ min) from control value. This resulted in a corresponding C.t-value of 0.2 $mg.min.m^{-3}$. In the present report, however, an extrapolated value for miosis was not taken into account.

2. *EEG*

Vehicle-pretreatment

The analysis of the online registered EEG epochs from vehicle-pretreated guinea pigs (n = 6) during a 5 h exposure to air is demonstrated in Fig 24. The averaged amounts of energy per band (d_1, d_2, t_1 etc.) did not change significantly between t = 0 and t = 300 min of exposure.

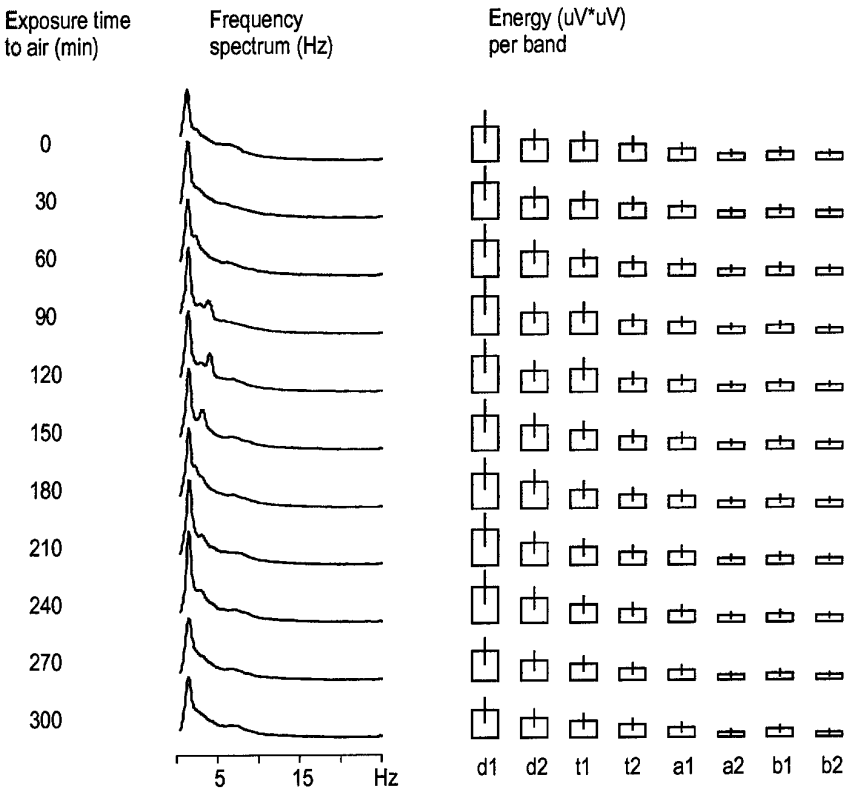

*Fig 24. EEG analysis of guinea pigs (n = 6) provided with Alzet pumps containing vehicle and exposed to air for 5 h. Indicated are the exposure time intervals at which the EEG-analysis was carried out, the averaged frequency spectrum and the averaged energy (μV*μV) per EEG-band per time interval.*

These averaged amounts of energy per EEG band of vehicle-pretreated and air-exposed animals were compared with the amounts of energy of the corresponding EEG bands of vehicle-pretreated and GB-exposed animals. In Table 11 only the EEG bands which appeared to be significantly different ($p < 0.05$) from that in air-exposed animals are given. It appeared that changes in the a-bands were predominant during the 5 h exposure of vehicle-pretreated animals to various concentrations of GB vapor. For all significant EEG-changes given in Table 11, the *LOAEL* (C.t) values were calculated using the actual concentrations (not shown) instead of the indicated mean concentrations in the table. These *LOAEL* values are given in Fig 25.

Table 11. Statistically analyzed differences between EEG-bands from vehicle-pretreated and GB-exposed (7.1, 14.3, 22.9, 56.4, or 146.2 µg/m³ GB, one animal per concentration) guinea pigs, and the corresponding EEG-bands from vehicle-pretreated and air-exposed (n = 6) animals. Indicated are the EEG-bands which are significantly different (p<0.05) from the corresponding bands in air exposed animals.

Mean GB (5h) conc. (µg/m³)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.1 ± 0.5							a_1		a_1	a_1 a_2
14.3 ± 0.5		a_1 a_2 b_1		a_1				a_1	a_1	a_1
22.9 ± 0.5	a_1 a_2 b_1	a_1 a_2 b_1		d_2			a_1	a_1	a_1	a_1
56.4 ± 1.0	a_1	a_2 b_1 b_2	a_1							
146.2 ± 1.9		a_1 a_2					a_1			

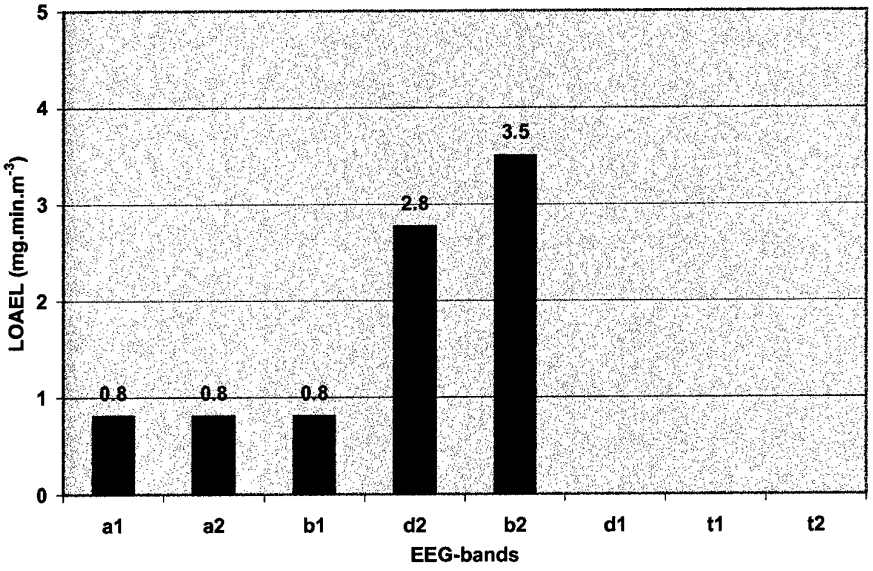

Fig 25. EEG-bands (horizontal axis) of vehicle-pretreated GB-exposed guinea pigs which became first significantly (p < 0.05) different from the corresponding bands in air exposed animals, and the calculated corresponding LOAEL levels (vertical axis).

It appeared that the a_1, a_2 and b_1 EEG-bands were most sensitive to GB exposure, whereas the d_2 and b_2 bands were least sensitive. There were no EEG changes regarding d_1, t_1 and t_2 bands. The lowest C.t-value established in this way was 0.8 mg.min.m^{-3} (at t = 60 min), representing the *LOAEL* for the first emerging EEG changes (a_1, a_2 and b_1 bands) in vehicle-pretreated and GB-exposed guinea pigs.

Pyridostigmine-pretreatment

The analysis of the online registered EEG epochs from pyridostigmine-pretreated guinea pigs (n = 6) during a 5 h exposure to air is demonstrated in Fig 26. The averaged amounts of energy per band (d_1, d_2, t_1 etc.) did not change significantly between t = 0 and t = 300 min of exposure.

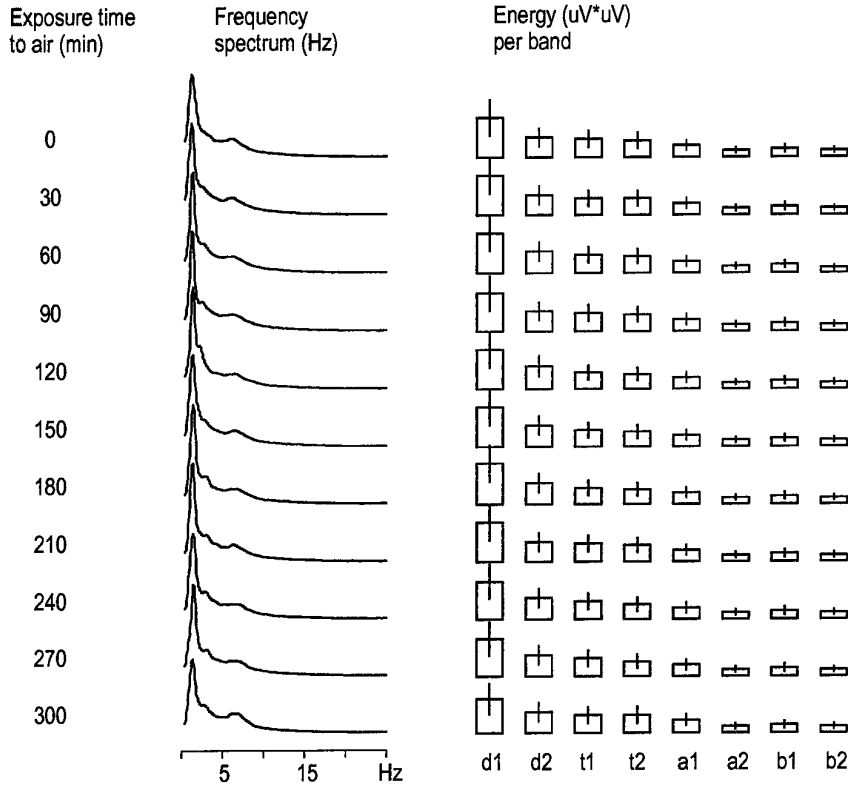

*Fig 26. EEG analysis of guinea pigs (n = 6) provided with Alzet pumps containing pyridostigmine (0.04 mg/kg/h) and exposed to air for 5 h. Indicated are the exposure time intervals at which the EEG-analysis was carried out, the averaged frequency spectrum and the averaged energy (μV*μV) per EEG-band per time interval.*

These averaged amounts of energy per EEG band of pyridostigmine-pretreated and air-exposed and animals were compared with the amounts of energy of the corresponding EEG bands of pyridostigmine-pretreated and GB-exposed animals. In Table 12 only the EEG bands are given which appeared to be significantly ($p < 0.05$) different from the corresponding bands in air-exposed animals.

Table 12. Statistically analysed differences between EEG-bands from pyridostigmine-pretreated and GB-exposed (11.5, 15.2, 24.2, 50.8, 151.3 or199.5 µg/m³ GB, one animal per concentration) guinea pigs, and the corresponding EEG-bands from pyridostigmine-pretreated and air-exposed (n = 6) animals. Indicated are the EEG bands which are significantly different (p < 0.05) from the corresponding bands in air exposed animals.

Mean GB (5h) conc. (µg/m³)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
11.5 ± 1.1			t_1						d_2	d_1 d_2 t_2
15.2 ± 0.1	d_1 d_2 t_2 a_1	t_2 a_1 a_2	t_2 a_1 b_2	a_1	d_1 t_1 a_1 b_2	d_1 a_1	a_1	t_1 a_1 a_2	d_2 t_1	a_1
24.2 ± 0.2	a_1	t_1 t_2 a_1 b_2	d_2 t_1 t_2 a_1 b_2	t_1 b_2	d_2 t_2 a_2 b_2	t_2	t_2		t_2	t_2
50.8 ± 0.5		a_2 b_2	b_2		b_2					
151.3 ± 1.2				a_1	a_1	a_1	a_1			
199.5 ± 2.3										t_1 b_2

Changes in the a- and t-bands were predominant during the 5 h exposure of pyridostigmine-pretreated animals to various concentrations of GB vapor.

For all significant EEG-changes given in Table 12, the *LOAEL* (C.t) values were calculated using the actual GB concentrations (not shown) instead of the indicated mean concentrations in the table. These *LOAEL* values are given in Fig 27. It appeared that the d_1, d_2, t_2 and a_1 EEG-bands were most sensitive to GB exposure. The lowest C.t-value established in this way was 0.4 mg.min.m^{-3} (at t = 30 min), representing the *LOAEL* for the first emerging EEG changes (bands d_1, d_2, t_2 and a_1) in pyridostigmine-pretreated and GB-exposed guinea pigs.

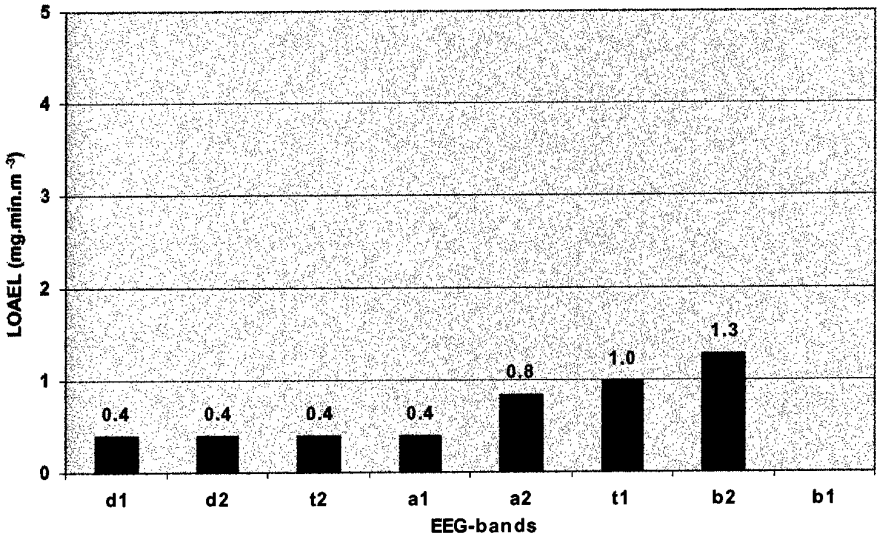

Fig 27. EEG-bands of pyridostigmine-pretreated GB-exposed guinea pigs which became first significantly ($p < 0.05$) different from the corresponding bands in air exposed animals (horizontal axis), and the calculated corresponding LOAEL levels (vertical axis).

Fig 28 shows the differences in *LOAEL* (C.t) levels between vehicle or pyridostigmine-pretreated GB-exposed animals regarding the first changing EEG-bands. The d_2, a_1 and b_2 bands in pyridostigmine-pretreated animals are more sensitive for GB than the corresponding bands in vehicle-pretreated animals. In vehicle-pretreated animals d_1, t_1, t_2 and b_1 bands did not change upon GB exposure.

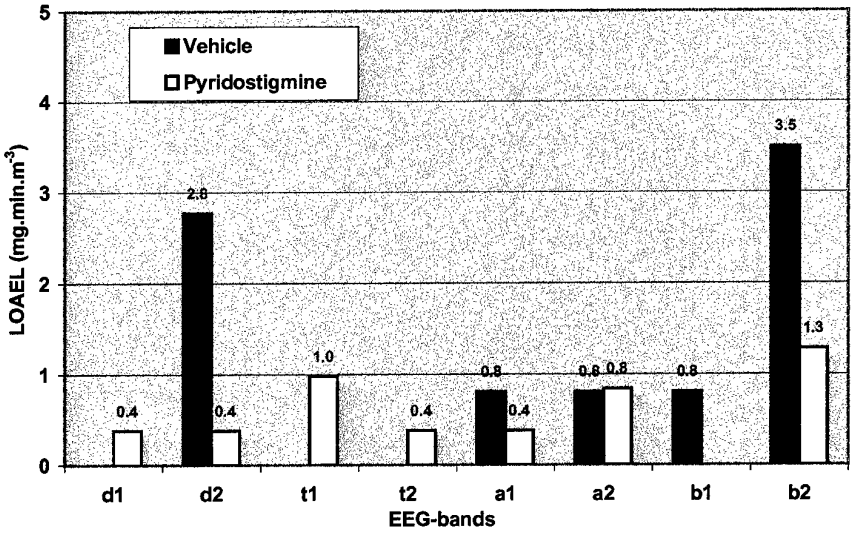

Fig 28. Comparison of EEG-bands (horizontal axis) of vehicle-pretreated (blue) or pyridostigmine-pretreated (white) GB-exposed guinea pigs which became first significantly ($p < 0.05$) different from the corresponding bands in air exposed animals, and of the calculated corresponding LOAEL levels (vertical axis).

3. *Visual-evoked response (VER)*

Vehicle-pretreatment

The VER latency parameters t_1, t_2, t_3 and t_4 obtained from restrained conscious and vehicle-pretreated guinea pigs at 1, 2, 3, 4 h and at the end of a 5 h exposure to various concentrations of GB, differed significantly ($p < 0.05$) from the averaged values of the corresponding latencies measured in vehicle-pretreated animals ($n = 6$) at the same intervals of exposure to air (Table 13). In this table the mean GB concentrations during the 5 h exposure period are given. For calculating the *LOAEL* (C.t) at t = 120 min, however, the mean GB concentration between t = 0 and t =120 min was used (6.6 $\mu g/m^3$), which resulted in a *LOAEL* of 0.8 $mg.min.m^{-3}$ for the VER in vehicle-pretreated animals.

Table 13. Statistically analyzed differences between the VER-latencies $t_1 - t_4$ obtained on-line from restrained conscious and vehicle-pretreated guinea pigs during a 5 h exposure period to either air (n = 6) or various concentrations of GB vapor in air (7.1, 7.4, 14.3, 22.9, 56.4, or 146.2 $\mu g/m^3$, one animal per concentration). Indicated are the latencies which are significantly different ($p < 0.05$) from the corresponding latencies in air exposed animals.

Mean GB conc. [$\mu g/m^3$]	Exposure time (min)				
	60	120	180	240	300
7.1 ± 0.5		t_3		t_1	
14.3 ± 0.5		t_2	t_1	t_1	t_1 t_2
22.9 ± 0.5	t_2		t_1		
56.4 ± 1.0	t_2			t_1	
146.2 ± 1.9				t_1	

Pyridostigmine-pretreatment

The latency parameters t_1, t_2, t_3 and t_4 in the VER of restrained conscious and pyridostigmine-pretreated guinea pigs at 1, 2, 3, 4 h and at the end of a 5 h exposure to various concentrations of GB, were significantly ($p < 0.05$) different from the averaged values of the corresponding latencies measured in pyridostigmine-pretreated animals ($n = 6$) at the same intervals of exposure to air (Table 14). In this table the mean GB concentrations during the 5 h exposure period are given. For calculating the *LOAEL* (C.t) at t = 60 min, however, the mean GB concentration between t = 0 and t = 60 min was used (13.8 $\mu g/m^3$), which resulted in a *LOAEL* of 0.8 $mg.min.m^{-3}$ for the VER in pyridostigmine-pretreated animals.

Table 14. Statistically analyzed differences between the VER-latencies $t_1 - t_4$ obtained online from restrained conscious and pyridostigmine-pretreated guinea pigs during a 5 h exposure period to either air (n = 6) or various concentrations of GB vapor in air (11.5, 15.2, 24.2, 50.8, 151.3 or 199.5 $\mu g/m^3$, one animal per concentration). Indicated are the latencies which are significantly different ($p < 0.05$) from the corresponding latencies in air exposed animals.

Mean GB conc. [$\mu g/m^3$]	Exposure time (min)				
	60	120	180	240	300
11.5 ± 0.1				t_1	t_3 t_4
15.2 ± 0.1	t_1				
24.2 ± 0.2	t_3 t_4	t_3 t_4	t_3	t_1	
50.8 ± 0.5					
151.3 ± 1.2	t_3 t_4	t_4	t_2 t_3 t_4	t_1 t_3	t_3
199.5 ± 2.3	t_1				

4. Startle-response

Vehicle-pretreatment

It was investigated whether the startle-response of an individual guinea pig after a 5 h exposure to GB would differ significantly ($p < 0.05$) from that (mean ± SEM) of a group of animals ($n = 6$) after a 5 h of exposure to air. The animals own control values were not taken into account. The startle-response of vehicle-pretreated guinea pigs (Gp61-66) at the end of a 5 h exposure to either 7.1, 14.3, 25, 22.9, 56.4 or 146.2 µg/m^3 GB vapor (one animal per concentration) did not differ significantly ($p > 0.05$) from the averaged startle-response measured in vehicle-pretreated animals (Gp25-30) ($n = 6$) at the end of a 5 h exposure to air (Fig 29).

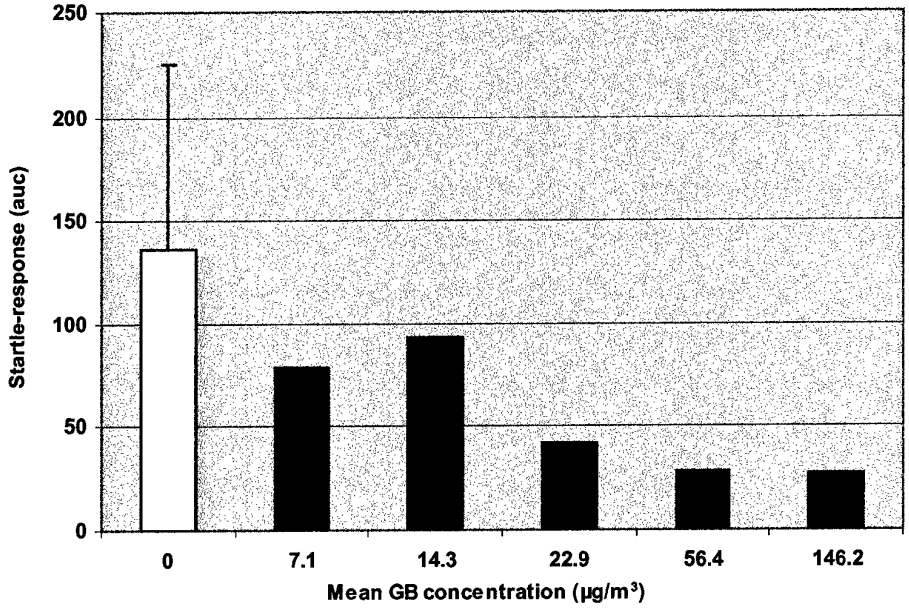

Fig 29. Startle-response of vehicle-pretreated guinea pigs at the end of a 5 h exposure to air (n = 6)(mean ± SEM) or to various mean concentrations of GB (7.1, 14.3, 22.9, 56.4, 146.2 µg/m^3, one animal per concentration).

None of the GB concentrations tested resulted in significant effects on the startle-response after a 5 h exposure period was > 146.2 µg/m^3. The *LOAEL* (C.t-value) for the startle-response in vehicle-pretreated animals will therefore be > 44 mg.min.m^{-3}.

Pyridostigmine-pretreatment

The startle-response of pyridostigmine-pretreated animals (Gp67-72) at the end of a 5 h exposure to either 11.5, 15.2, 24.2, 50.8, 151.3 or 199.5 µg/m^3 GB vapor did not differ significantly from the averaged startle-response measured in pyridostigmine-pretreated animals (Gp31-36) (n = 6) at the end of a 5 h exposure to air. As an exception, exposure to 50.8 µg/m^3 GB resulted in a significant ($p < 0.05$) increase in startle-response (Fig 30). Therefore, the *LOAEL* (C.t-value) for the startle-response in pyridostigmine-pretreated animals was calculated to be 15.2 mg.min.m^{-3}. Although we have no explanation for the observation that there was only an effect at 50.8 µg/m^3, the concentration range tested may be considered as a threshold range for emerging effects on startle-response.

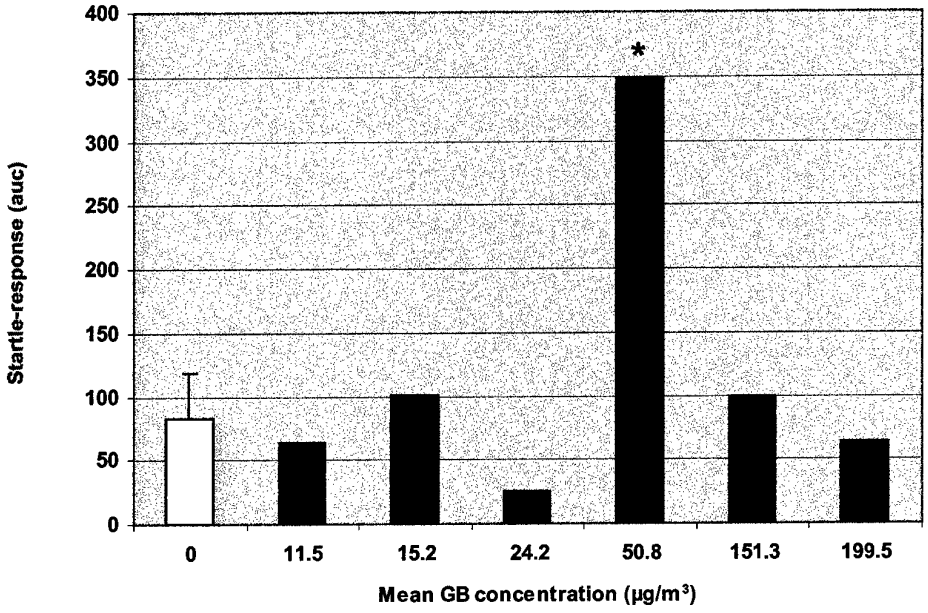

Fig 30. Startle-response of pyridostigmine-pretreated guinea pigs at the end of a 5 h exposure to air (n = 6)(mean ± SEM) or to various mean concentrations of GB (11.5 15.2, 24.2, 5.80, 151.3, 199.5 µg/m^3, one animal per concentration). (, significantly different from the mean value of the air-exposed animals, $p < 0.05$).*

5. Shuttle-box

Vehicle-pretreatment

After 8-10 training sessions all guinea pigs (Gp61-66) demonstrated between 90 and 100% correct responses in the shuttle-box (Fig 31). Four days after providing 6 of these animals with Alzet pumps containing vehicle, their performance was still 90% (session 11) which was not significantly ($p > 0.05$) different from that observed in session 10. Four days after providing the other 6 animals with Alzet pumps containing pyridostigmine, their performance was significantly ($p < 0.05$) lower than that in session 10.

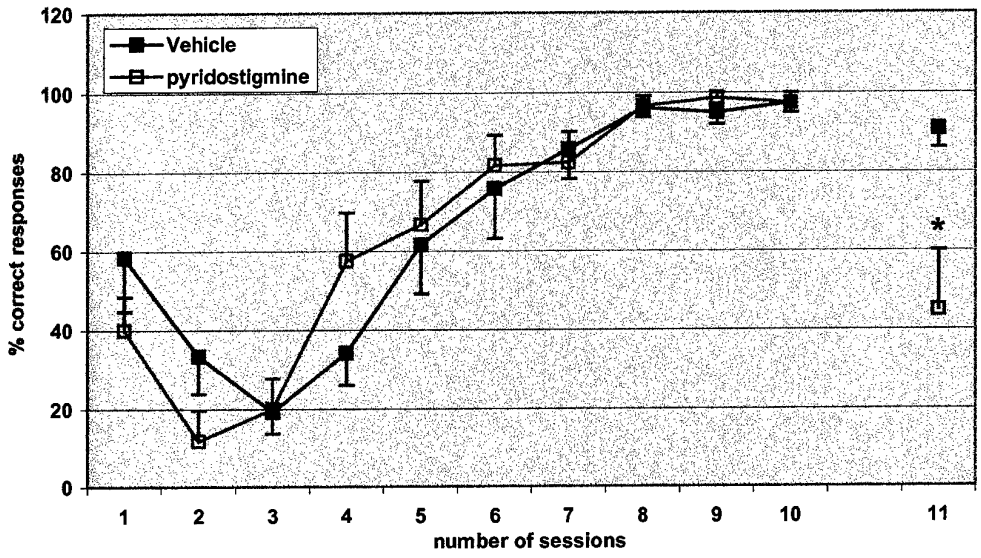

Fig 31. Training curves of two groups of each 6 guinea pigs to learn active avoidance behavior in a shuttle-box. After 8-10 training sessions, at the end of which 90-100% correct responses were gained by both groups, 6 animals received Alzet pumps containing vehicle (subcutaneously), the other 6 animals Alzet pumps containing pyridostigmine (0.04 mg/kg/h). Four days later (session 11) both groups were tested again. (, significant, $p < 0.05$, lower performance in session 11 than in session 10.*

A 5 h exposure of the vehicle-pretreated animals to either 7.1, 22.9, 56.4, 56.4 or 146.2 $\mu g/m^3$ GB vapor resulted in a significant ($p < 0.05$) decrease in performance compared to a 5 h exposure of vehicle-pretreated animals to air (Fig 32).

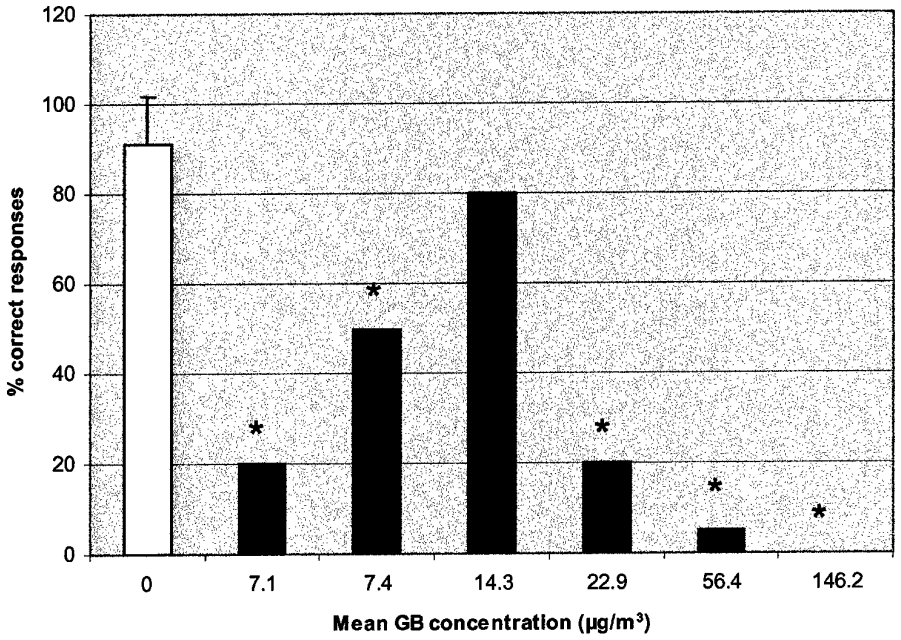

Fig 32. Shuttle-box performance of guinea pigs provided with Alzet pumps containing vehicle, after a 5 h exposure to either air (n = 6) (white bar) or various mean concentrations of GB (shaded bars, one animal per concentration). (, significantly ($p < 0.05$) different from the mean at GB concentration zero).*

The lowest GB concentration which resulted in significant effects on the shuttle-box behavior after a 5 h exposure period was 7.1 $\mu g/m^3$. This results in a *LOAEL* (C.t-value) of 2.1 $mg.min.m^{-3}$ for shuttle-box behavior in vehicle-pretreated animals.

Pyridostigmine-pretreatment

In contrast, a 5 h exposure of the pyridostigmine-pretreated (Gp67-72) animals to various concentrations of GB did not result in significant decreases in performance, except for the highest concentration of GB tested (199.5 $\mu g/m^3$) (Fig 33).

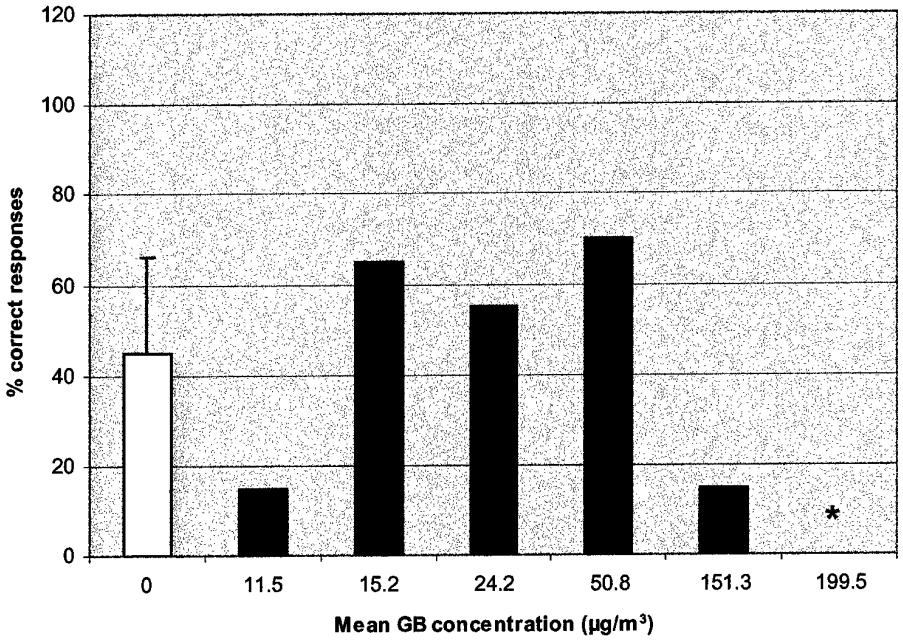

Fig 33. Shuttle-box performance of guinea pigs provided with Alzet pumps containing pyridostigmine, after a 5 h exposure to either air (n = 6) (white bar) or various mean concentrations of GB (red bar, one animal per concentration). (, significantly ($p < 0.05$) different from the mean).*

The lowest GB concentration which resulted in significant effects on shuttle-box behavior after a 5 h exposure period was 199.5 $\mu g/m^3$. This would result in a *LOAEL* (C.t-value) of 60 $mg.min.m^{-3}$ for shuttle-box performance in pyridostigmine-pretreated animals. It is not clear whether this finding should be considered as a protection by pyridostigmine since pyridostigmine by itself had depressed the shuttle-box behavior.

Acetylcholinesterase (AChE) activity in blood

Vehicle-pretreatment

A 5-h whole-body exposure to GB vapor concentrations in the range of 7.5 – 150 µg/m^3, did not result in significant ($p > 0.05$) decreases in AChE-activity in blood from vehicle-pretreated guinea pigs compared to the averaged AChE-activity (100%) in blood from animals ($n = 6$) at the end of a 5-h exposure to air (Fig 34). However, measurements of BuChE-inactivation based on release of the phosphyl moiety from the enzyme with fluoride ions (internal dose assessment) showed inhibition during exposures (compare Table 3), but at levels that can not be measured with the traditional AChE-activity measurements as given in Fig 34.

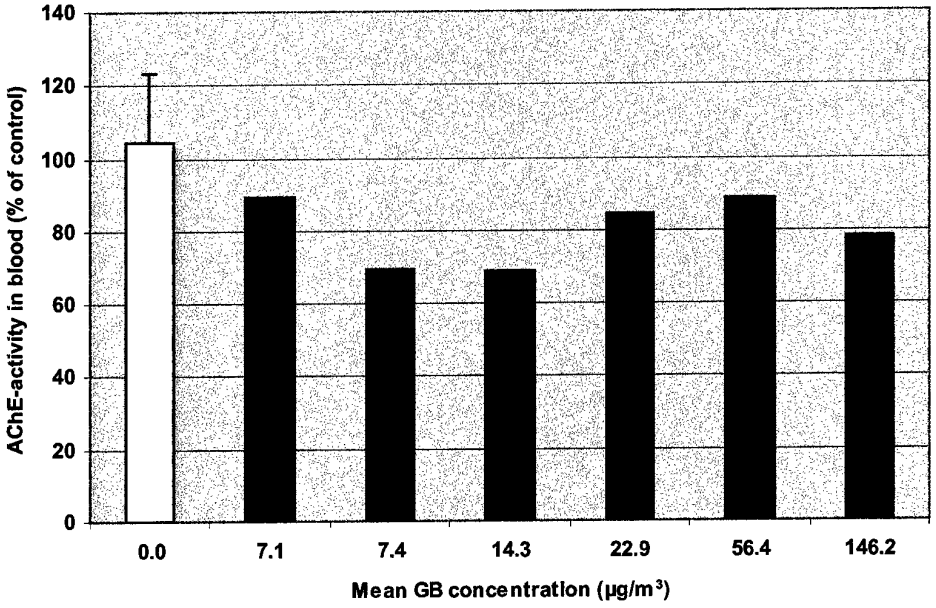

Fig 34. AChE-activity in blood from restrained conscious guinea pigs provided with Alzet pumps containing vehicle, determined at the end of a 5-h exposure to either air (n = 6) (mean ± SEM) (white bar) or to various mean concentrations of GB blue bar (one animal per concentration).

Pyridostigmine-pretreatment

The averaged AChE-activity in blood from pyridostigmine-pretreated animals (n = 6) before GB exposure was about 75% (i.e., 25% inhibition) of their own control values (Fig 35). A 5 h exposure to GB vapor concentrations in the range of 11.5 – 200 $\mu g/m^3$ did not result in significant further decreases in AChE-activity in blood of these animals.

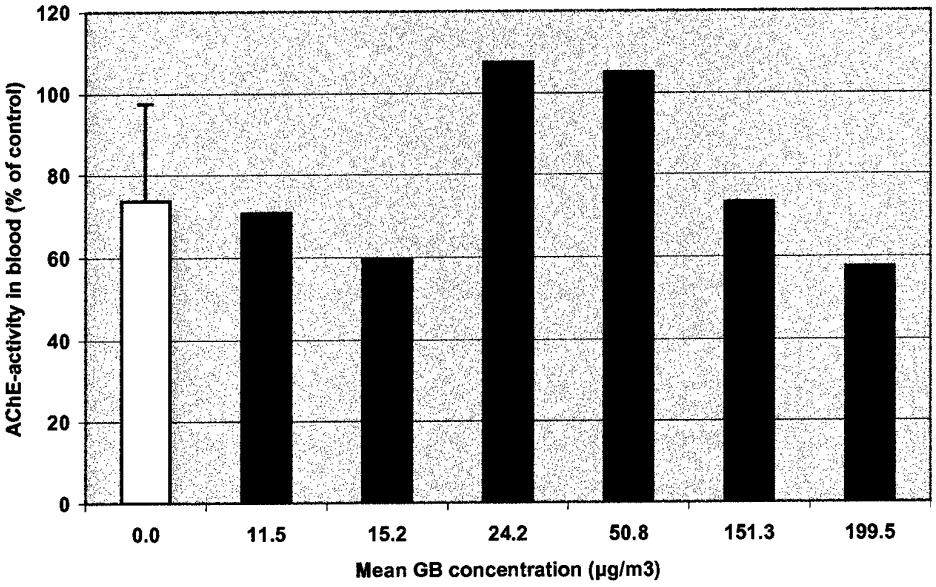

Fig 35. AChE-activity in blood from restrained conscious guinea pigs provided with Alzet pumps containing pyridostigmine, determined at the end of a 5 h exposure to either air (n = 6)(mean ± SEM) or to various mean concentrations of GB (one animal per concentration).

MARMOSET EXPERIMENTS

The Lowest Observable Effect Level (LOEL) of GB exposure for vehicle-pretreated marmosets

The generated GB vapor concentrations during the separate exposures of the individual vehicle-pretreated animals (M1 –M5) are given in Fig 36 (panels M1-M5). The generated GB concentrations in individual exposures was mostly within a range of 0.1 – 1.0 $\mu g/m^3$, except some short-lasting higher peaks at about t = 50 min (panel M2), at t = 75 min and t = 230 min (panel M4), respectively. Exposure periods needed to determine the associated GB internal dose in blood samples, differed between the animals from 30 – 180 min. The vertical bars indicate the first time points at which the internal doses could be detected in a reliable way, i.e. when the signal-to-noise ratio was equivalent to or greater than 2 ($S/N \geq 2$). The grey-shaded cells in Table 15 contain the internal doses in the course of the exposures which could be detected reliably and were taken into account for calculating the *LOEL*. Two or more subsequent reliable measurements of the internal GB dose in each animal were considered necessary for the assessment of the *LOEL*. Therefore, the first internal dose assessment (10.5 pg/ml) in marmoset M1 and that of 7.6 pg/ml in marmoset M5, were therefore not taken into account. The mean individual exposure concentrations of GB ($\mu g/m^3$) generated over the period of time between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected reliably (until vertical bar) are shown in Table 16. The individual *LOEL* levels of exposure were calculated on the basis of these values and were in the range between 0.022 – 0.066 $mg.min.m^{-3}$. The averaged *LOEL* level for vehicle-pretreated marmosets (M1 – M5) was calculated to be 0.04 ± 0.01 $mg.min.m^{-3}$ (Table 16).

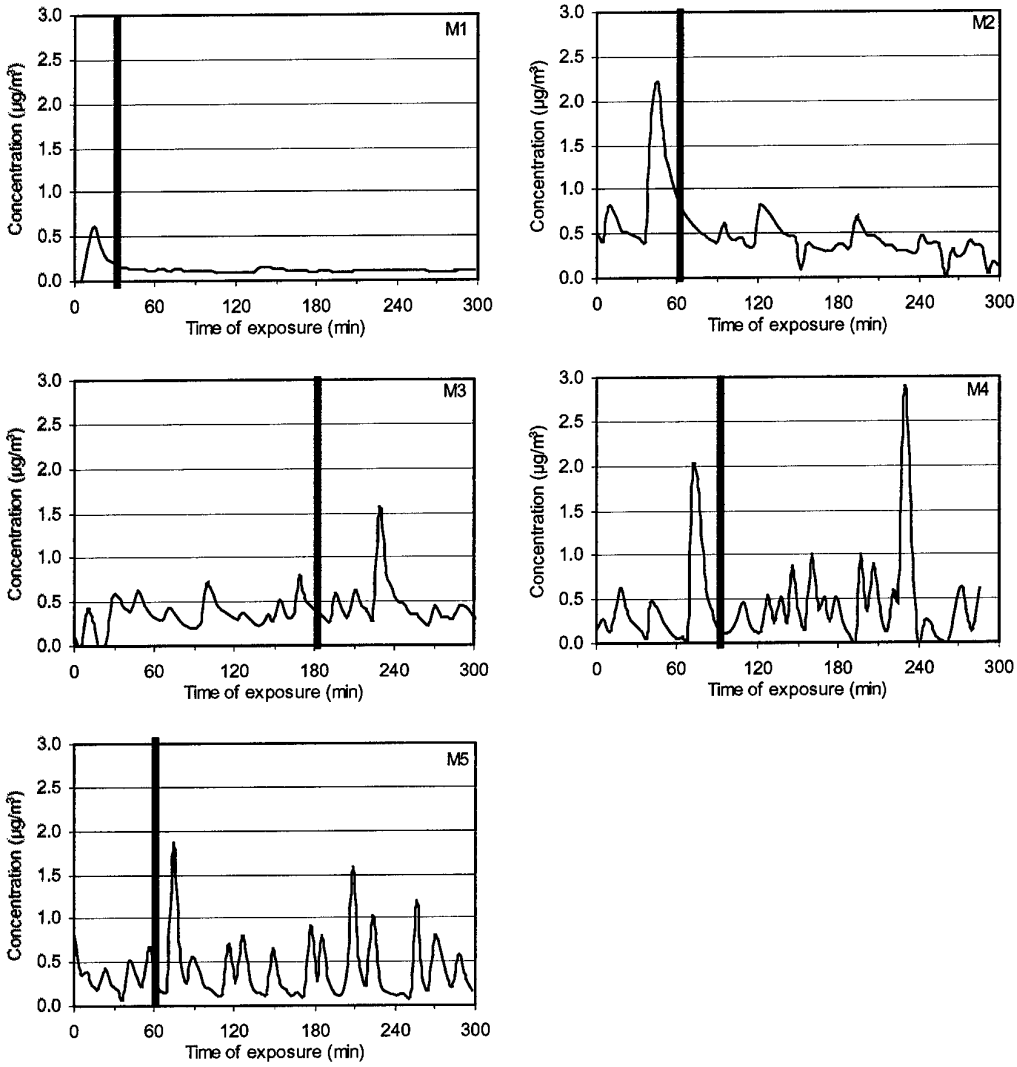

Fig 36. Time course (horizontal axis) of generated GB vapor concentrations (vertical axis) in air during exposure of vehicle-pretreated marmosets for determining LOEL levels. The vertical bars indicate the first time points at which the internal doses could reliably (S/N ≥ 2) be determined. The concentration of GB was measured semi-continuously at 2-5 min intervals.

Table 15. Fluoride-regenerated GB concentrations (pg/ml) determined in approximately 0.25 ml blood samples drawn from vehicle-pretreated marmosets (M1-M5) mainly exposed to 0.1 – 1.0 µg/m³ GB vapor in air. The shaded cells contain the first internal doses during the exposures which could be detected reliably (S/N > 2). Marmoset numbers (M) correspond to those shown in Fig 36. B.d. = below detection limit (S/N < 2); n.a. = not analyzed.

Time [min]	M1	M2	M3	M4	M5
0	*b.d.*	*b.d.*	*b.d.*	*b.d.*	7.6
30	10.5	*b.d.*	*n.a.*	*b.d.*	*b.d.*
60	*b.d.*	8.4	*n.a.*	*b.d.*	3.1
90	*b.d.*	5.3	*b.d.*	4.6	2.8
120	*n.a.*	8.9	*n.a.*	21.4	4.7
150	*n.a.*	11.2	*b.d.*	*n.a.*	*n.a.*
180	*n.a.*	12.8	8.8	*n.a.*	*n.a.*
210	*n.a.*	*n.a.*	5.3	*n.a.*	8.9
240	*b.d.*	*n.a.*	14.4	*n.a.*	*n.a.*
270	*b.d.*	*n.a.*	10.5	*n.a.*	*n.a.*
300	*b.d.*	18.5	12.4	71.3	19.9

Table 16. Mean exposure concentration of GB vapor in air (µg/m³) generated over the period of time (Time to LOEL) between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected reliably (S/N ≥ 2), and calculation of the individual LOEL (C.t) level of exposure . Marmoset numbers (M) correspond to those shown in Fig 36 and Table 15.

Marmoset	M1	M2	M3	M4	M5
Time to *LOEL* [min]	-	60	180	90	60
Mean (± sem)* GB vapor conc. to time to *LOEL* [µg/m³]	-	0.91 ± 0.16	0.36 ± 0.03	0.42 ± 0.11	0.33 ± 0.05
LOEL [mg.min.m^{-3}]	-	0.054	0.066	0.037	0.020
Mean (± sem) *LOEL* [mg.min.m^{-3}]	0.04 ± 0.01				

* *Time-based average of vapor concentrations measured at 2-5 min intervals.*

The Lowest Observable Effect Level (LOEL) of GB exposure for pyridostigmine-pretreated marmosets

The pyridostigmine-pretreated animals were numbered M6 – M10 (Fig 37). The inter-individual differences in generated GB concentrations were mostly in a range of 0.3 – 0.5 µg/m³ (see panel M6, 8, 9,10), except for marmoset M7 for which the exposure concentration was in a range of 0.1 – 1.0 µg/m³ (panel M7). The exposure period necessary to detect the associated GB internal doses in blood samples differed between the individual animals from 90 – 120 min. The shaded cells in Table 17 contain the first internal doses in the course of the individual exposures which could be detected reliably (S/N > 2) and were taken into account for calculating the *LOEL*. The mean individual exposure concentrations of GB (µg/m³) generated over the period of time between the start of the exposure and the time point at

which fluoride-regenerated GB (internal dose) could be detected reliably (S/N > 2) (until vertical bar in Fig 37) are shown in Table 18. The individual *LOEL* levels were calculated on the basis of these values and were in the range between 0.045 – 0.053 mg.min.m^{-3}. The first measurements in M5, M6, and M10 were not taken into consideration because of contamination of internal standard with GB (M10) and/or carry-over problem (M5 and M6). Such problems occur regrettably in the course of ultra-trace analysis.
The internal doses measured from t = 90 min on, were considered reliable and were taken into account for the assessment of the *LOEL*. The averaged *LOEL* level for pyridostigmine-pretreated marmosets (M6 – M10) was calculated to be 0.050 ± 0.002 mg.min.m^{-3} (Table 18) and was statistically not significantly different (p > 0.05) from that of vehicle-pretreated animals.

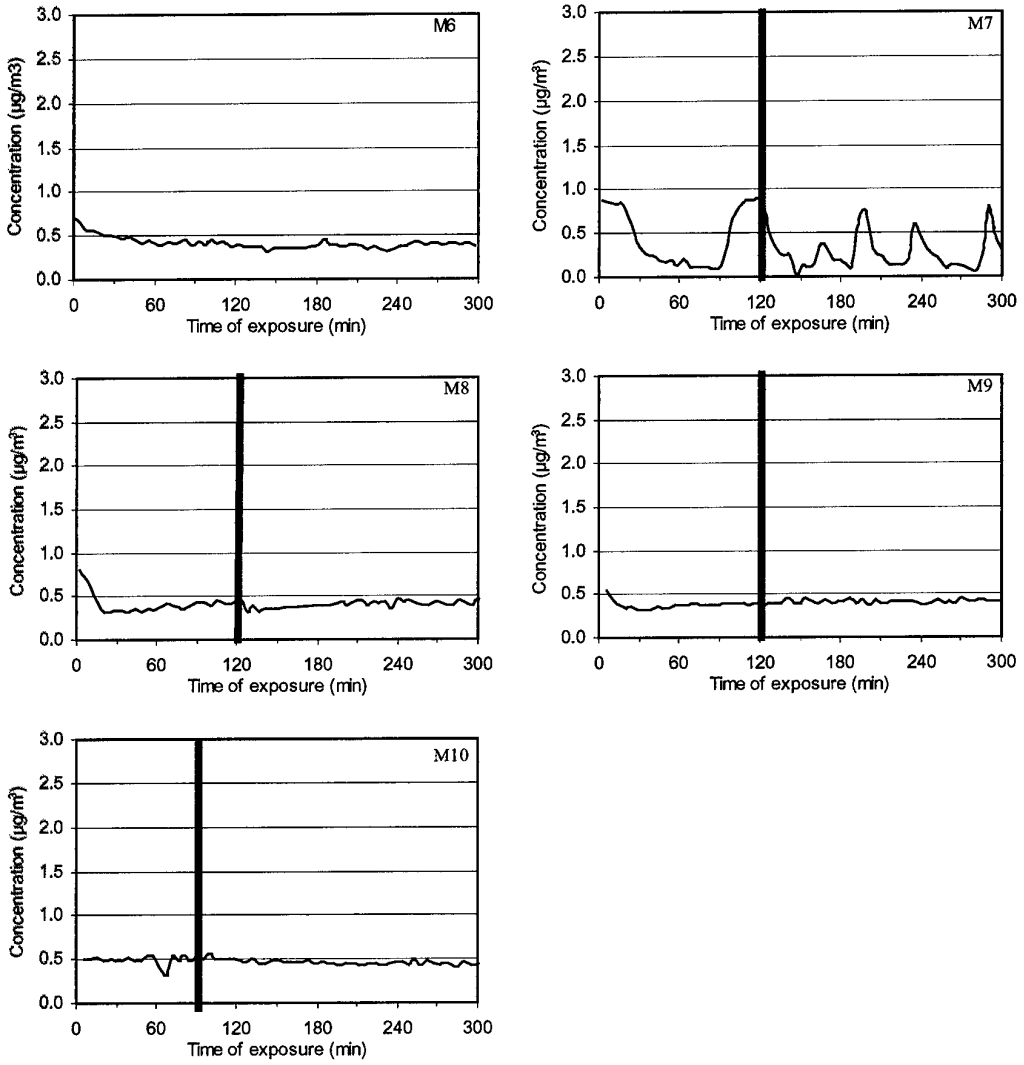

Fig 37. Time course (horizontal axis) of generated GB vapor concentrations (vertical axis) in air during exposure of pyridostigmine-pretreated marmosets for determining LOEL levels. The vertical bars indicate the first time points at which the internal doses could reliably (S/N ≥ 2) be detected.

Table 17. Fluoride-regenerated GB concentrations (pg/ml) determined in 0.5 ml blood samples drawn from pyridostigmine-pretreated marmosets (M6-M10) exposed to 0.1 – 1.0 µg/m³ GB vapor in air. The shaded cells contain the first internal doses during the exposures which could be detected in a reliable (S/N ≥ 2) way. Marmoset numbers (M) correspond to those shown in Fig 37. B.d. = below detection limit (S/N < 2); n.a. = not analyzed.

Time [min]	M6	M7	M8	M9	M10
0	5.5	*b.d.*	*b.d.*	*b.d.*	5.9
30	7.8	*b.d.*	*b.d.*	*n.a.*	24.8
60	8.1	*n.a.*	*b.d.*	*b.d.*	*b.d.*
90	14.4	*b.d.*	*b.d.*	*b.d.*	8.7
120	*n.a.*	3.8	5.1	3.6	69.4
150	*n.a.*	3.9	26.5	*b.d.*	9.7
180	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
210	*n.a.*	*n.a.*	*n.a.*	4.1	*n.a.*
240	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
270	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
300	26.0	12.4	12.2	4.0	21.1

Table 18. Mean exposure concentration of GB vapor in air (µg/m³) generated over the period of time (LOEL-time) between the start of the exposure and the time point at which fluoride-regenerated GB (internal dose) could be detected in a reliable (S/N ≥ 2) way, and calculation of the individual LOEL. Marmoset numbers (M) correspond to those shown in Fig 37 and Table 17.

Marmoset	M6	M7	M8	M9	M10
Time to *LOEL* [min]	-	120	120	120	90
Mean (± sem)* GB vapor Conc. To time to *LOEL* [µg/m³]	-	0.44 ± 0.06	0.42 ± 0.02	0.38 ± 0.01	0.49 ± 0.01
LOEL [mg.min.m^{-3}]	-	0.053	0.050	0.045	0.045
Mean (± sem) *LOEL* [mg.min.m^{-3}]	0.05 ± 0.002				

**Time-based average of vapor concentrations measured at 2-5 min intervals.*

The Lowest Observable Adverse Effect Levels (LOAEL) of GB exposure for marmosets

Vehicle-pretreatment

The GB vapor concentrations generated to determine the *LOAEL* levels (C.t) of exposure at which significant ($p < 0.05$) changes are expected to emerge regarding pupil size, EEG, VER, startle-response, and bungalow-test behavior of vehicle-pretreated marmosets, are shown in Fig 38. The aim was to expose animals during 5 h exposure periods to the following concentrations of GB: 150, 50, 25, 15 and 7.5 µg/ m^3, one animal per concentration. The first animal was exposed to the highest concentration (150 µg/ m^3), the second to 50 µg/ m^3, etc. The calculated mean concentrations that were actually achieved are given in Table 18.

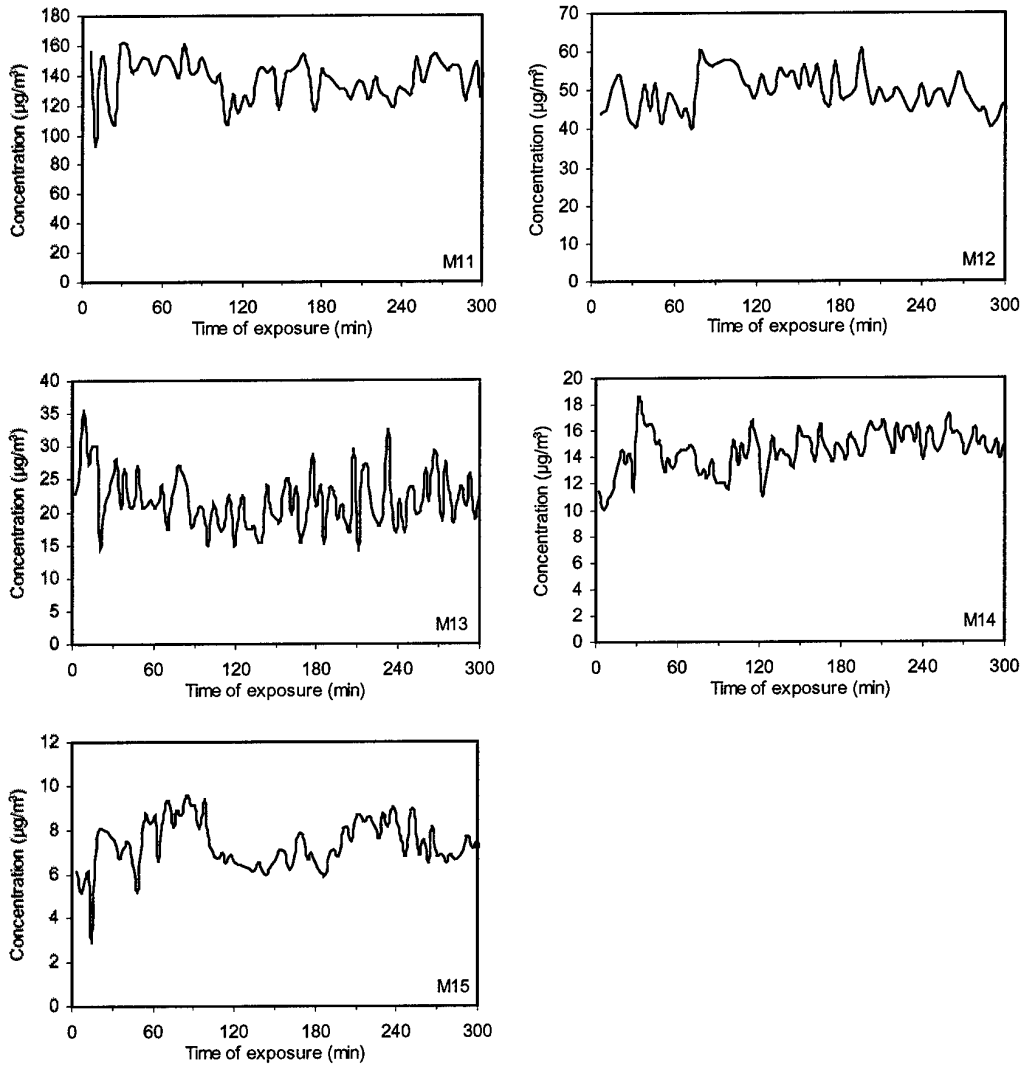

Fig. 38. Time course (min, horizontal axis) of generated GB vapor concentrations(µg/m^3) in air (vertical axis) during exposure of vehicle-pretreated marmosets for determination of LOAEL levels. The concentration of GB was semi-continuously measured at 2-5 min intervals.

Table 18. Calculated actual mean concentrations of GB vapor to which vehicle-pretreated marmosets were exposed in order to determine the LOAEL (C.t) levels of exposure.

Marmoset no.	Mean (± sem)* GB concentration ($\mu g/m^3$) between t = 0 and t = 300 min
M11	137.7 ± 1.7
M12	49.7 ± 0.6
M13	21.8 ± 0.4
M14	14.6 ± 0.2
M15	7.3 ± 0.1

**Time-based average of vapor concentrations measured at 2-5 min intervals.*

Pyridostigmine-pretreatment

The vapor concentrations of GB that were generated to determine the *LOAEL* levels (C.t) of exposure at which significant ($p < 0.05$) changes are expected to emerge regarding pupil size, EEG, VER, startle-response, and bungalow-test behavior of pyridostigmine-pretreated marmosets, are shown in Fig 39. It was intended to produce the following concentrations: 150, 50, 25, 15 and 7.5 $\mu g/m^3$. The first animal was exposed to 150 $\mu g/m^3$, the second to the next lower concentration: 50 $\mu g/m^3$, etc. (one animal per concentration). The calculated mean concentrations that were actually achieved are given in Table 19.

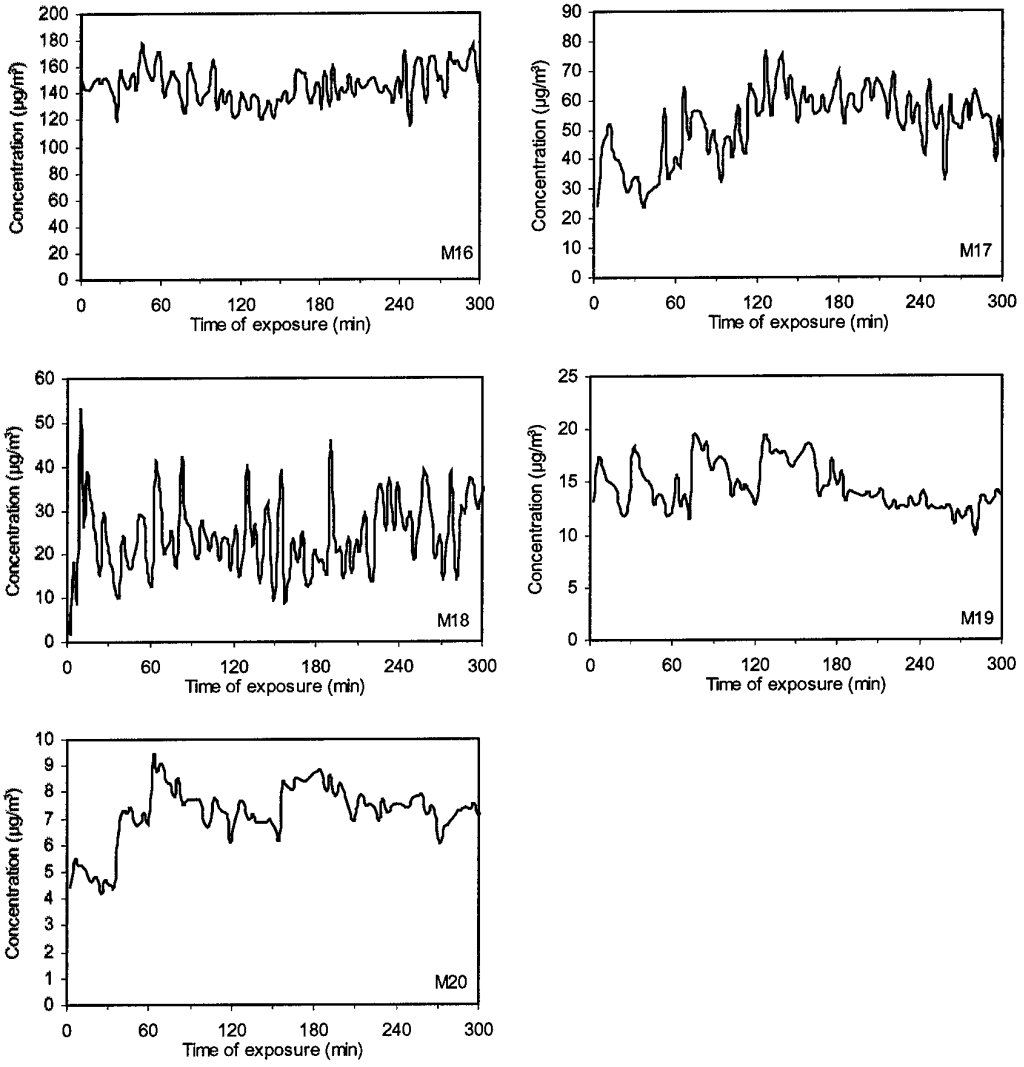

Fig 39. Time course (horizontal axis) of generated vapor concentrations of GB ($\mu g/m^3$) in air (vertical axis) during exposure of pyridostigmine-pretreated marmosets for determining LOAEL levels. The concentration of GB was semi-continuously measured at 2-5 min intervals.

Table 19. Calculated actual mean concentrations of GB vapor to which pyridostigmine-pretreated marmosets were exposed in order to determine the LOAEL (C.t) levels of exposure.

Marmoset no.	Mean (± SEM)* GB concentration ($\mu g/m^3$) between t = 0 and t = 300 min
M16	145.9 ± 1.3
M17	51.7 ± 1.2
M18	24.2 ± 0.9
M19	14.6 ± 0.2
M20	7.2 ± 0.1

**Time-based average of vapor concentrations measured at 2-5 min intervals*

The concentrations of fluoride-regenerated GB determined for vehicle- and pyridostigmine-pretreated marmosets in blood samples taken at the end of the 5 h exposure to various doses of GB vapor in air, are demonstrated in Table 20. The relationship between these two parameters is given in Fig 40. It appeared that pyridostigmine-pretreatment resulted in lower concentrations of bound GB in blood, at least at the end of a 5 h whole-body exposure to various concentrations of GB vapor.

Table 20. Doses ($mg.min.m^{-3}$), and concentrations (ng/ml) of fluoride-regenerated GB in blood samples taken from marmosets at the end of the 5 h exposure to various doses of GB. M11-M15: vehicle-pretreated marmosets; M16- M20: pyridostigmine-pretreated animals.

Marmoset			
Vehicle	Pyridostigmine	Dose [$mg.min/m^3$]	Fluoride-regenerated GB in blood [ng/ml]
M11		41.3	14.8
M12		14.9	9.2
M13		6.5	4.0
M14		4.4	*no sample*
M15		2.2	2.0
	M16	43.8	11.2
	M17	15.5	5.1
	M18	7.3	3.6
	M19	4.4	4.0
	M20	2.2	1.9

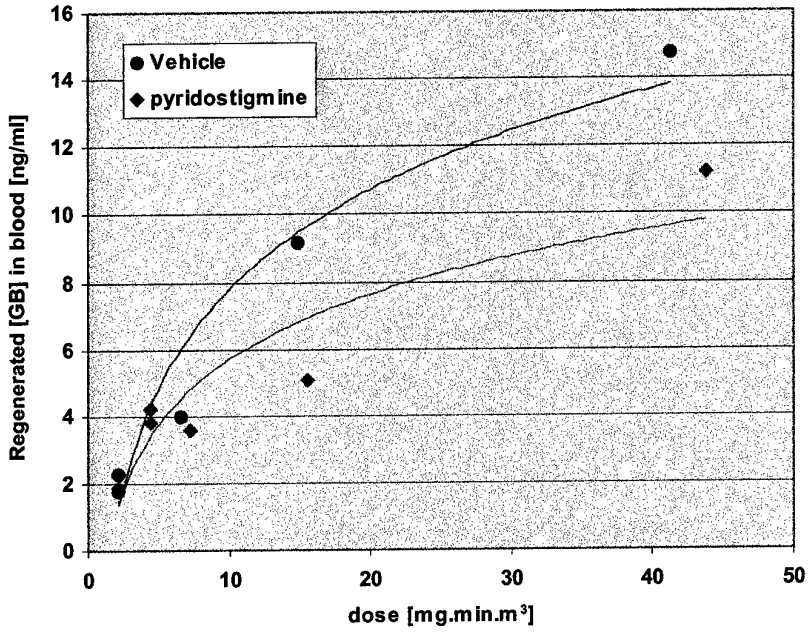

Fig 40. Relationship between concentrations of fluoride-regenerated GB (ng/ml) in blood at the end of a 5 h exposure of marmosets to GB concentrations in the range of 7.5-150 μg/m^3, and dose levels (mg.min.m^{-3}).

1. *Miosis*

Vehicle-pretreatment

A 5 h whole-body exposure of vehicle-pretreated marmosets to GB vapor concentrations in the range of 7.3 – 137.7 μg/m^3, resulted in significant ($p < 0.05$) and concentration-related decreases in pupil size (miosis) compared to the averaged control pupil size (0.67) in animals ($n = 5$) at the end of a 5-h exposure to air (Fig 41). A decrease of about 10% in pupil size was significantly ($p < 0.05$) different from control value.

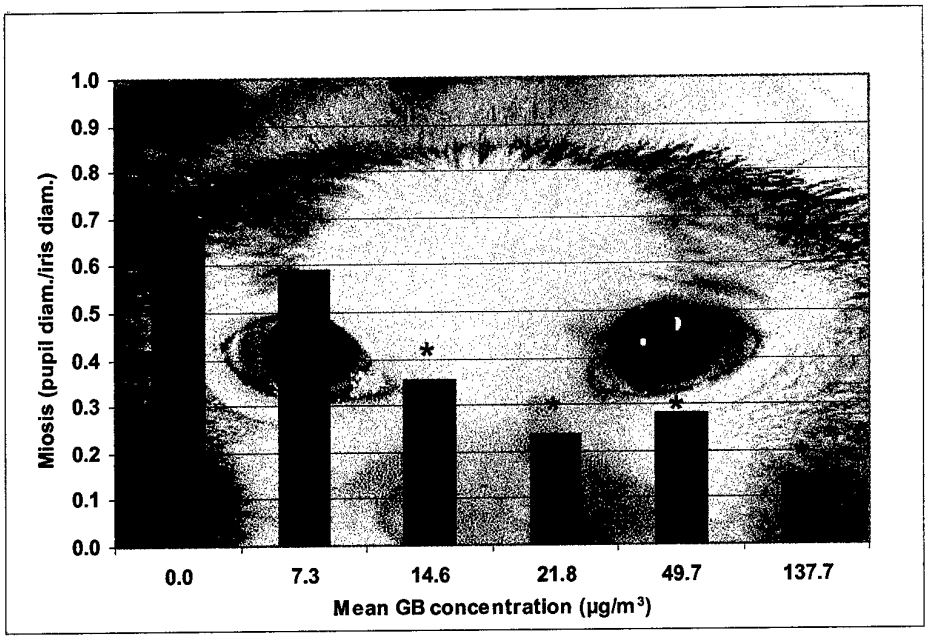

Fig 41. Miosis of 5 restrained conscious marmosets provided with Alzet pumps containing vehicle, after a 5 h exposure to either air (0.0, control, mean ± SEM), and after a 5 h exposure to various mean concentrations of GB (one animal per concentration). (, significantly different from control, p < 0.05)*

In Table 22 the exposure times needed to achieve significant ($p < 0.05$) miosis during exposure to the various mean GB concentrations and the corresponding C.t values are given. At a GB concentration of 7.3 μg/m^3 the decrease in pupil size became significant ($p < 0.05$) during the 5th h of exposure, whereas at the higher concentrations (21.8 – 137.7 μg/m^3), miosis was manifest much earlier during the 5 h exposure period, see Fig 42 for a typical example.

The mean C.t value (Table 22) was taken as the *LOAEL* regarding miosis in vehicle-pretreated marmosets albeit at various exposure times: 2.5 ± 0.8 mg.min.m^{-3}.

The relationship between the degree of miosis at the end of the 5 h exposure period and the mean GB exposure concentrations is given on a linear scale in Fig 43.

Table 22. Exposure times needed to achieve significant ($p < 0.05$) miosis during exposure to the various mean GB concentrations, and the corresponding C.t value. The concentrations given in the table are the actual mean concentrations between t = 0 and the time point at which miosis became significantly different from the control value. Nm = not measured.

Vehicle-pretreatment		
Mean (± sem) conc. of GB exposure ($\mu g/m^3$)	Time (min) to significant miosis ($p<0.05$)	C.t ($mg.min.m^{-3}$)
-	Nm	-
13.9 ± 0.3	121	1.68
23.7 ± 0.8	87	2.07
49.3 ± 1.4	100	4.93
124.4 ± 31.7	11	1.36
	Mean ± sem	2.5 ± 0.8

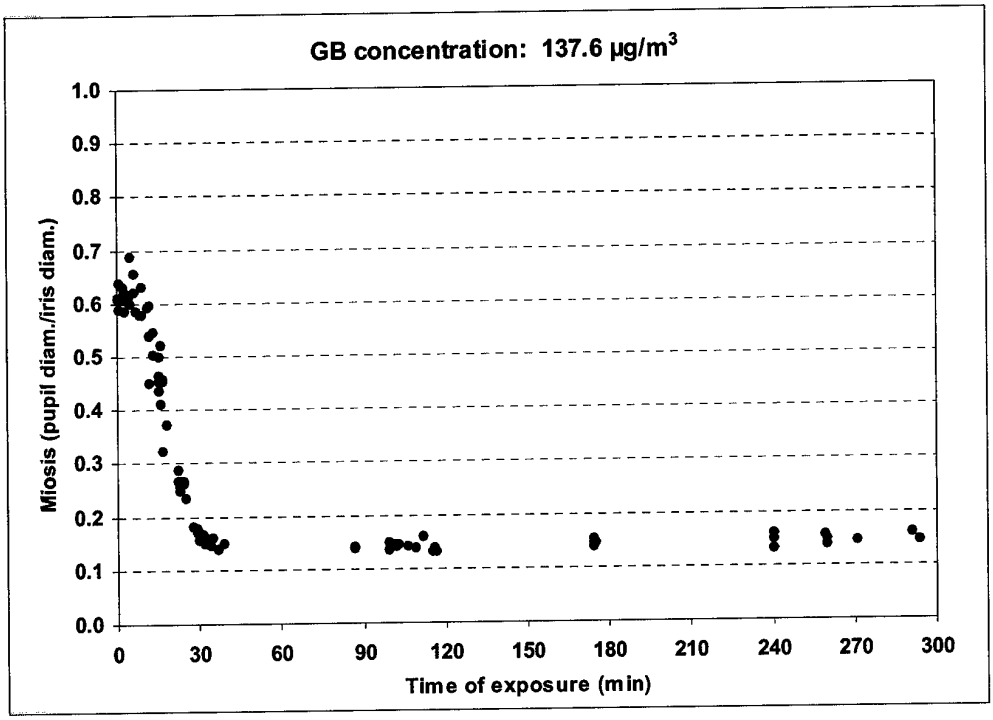

Fig 42. Development of miosis in a vehicle-pretreated marmoset M11 which was whole-body exposed to a mean concentration of 137.7 $\mu g/m^3$ of GB vapor in air during a 5 h exposure period. Compare Fig 41).

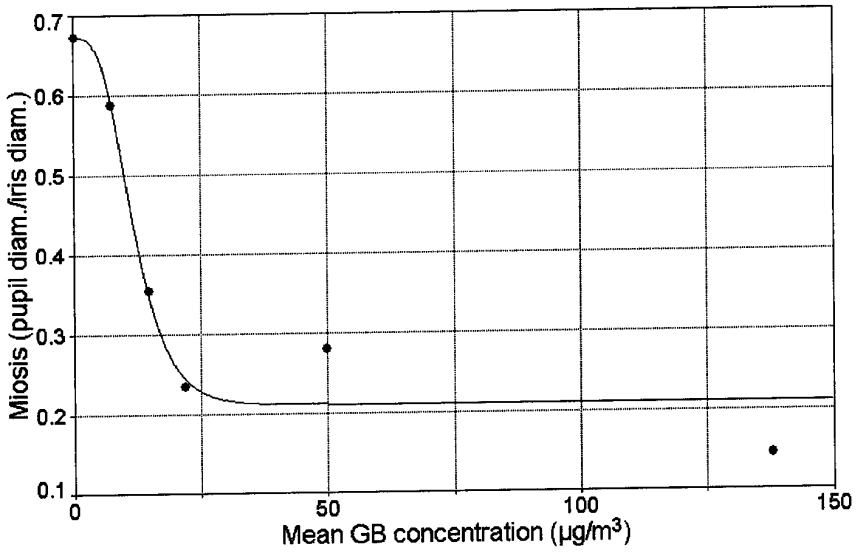

Fig 43. Relationship between the degree of miosis at the end of a 5 h exposure to various mean concentrations of GB, and the GB exposure concentrations for vehicle-pretreated marmosets. The points to be fitted were taken from Fig 41.

Pyridostigmine-pretreatment

A 5 h exposure of pyridostigmine-pretreated animals to GB vapor concentrations (7.2 – 145.9 $\mu g/m^3$), resulted in significant ($p < 0.05$) and concentration-related decreases in pupil size (miosis) compared to the averaged pupil size (0.68) in animals at the end of a 5 h exposure to air (control, n = 5) (Fig 44). A decrease of about 10% in pupil size was significantly ($p < 0.05$) different from control value. During exposure to a GB concentration of 14.6 $\mu g/m^3$, miosis became significant ($p < 0.05$) during the 5th h of exposure. At the higher concentrations (24.2 – 145.9 $\mu g/m^3$), miosis was manifest much earlier during exposure (not shown), except at the concentration of 24.2 $\mu g/m^3$. The animal exposed to the latter mean concentration kept its eyes closed during exposure thereby precluding exposure to GB.

In Table 23 the exposure times needed to achieve significant ($p < 0.05$) miosis during exposure to the various mean concentrations and the corresponding C.t values are given. The mean C.t value was taken as the *LOAEL* regarding miosis in pyridostigmine-pretreated marmosets albeit that the values were obtained at various exposure times: 3.0 ± 0.8 $mg.min.m^{-3}$.

The relationship between the degree of miosis in pyridostigmine-pretreated animals and the GB exposure concentrations was fitted on a linear scale in Fig 45.

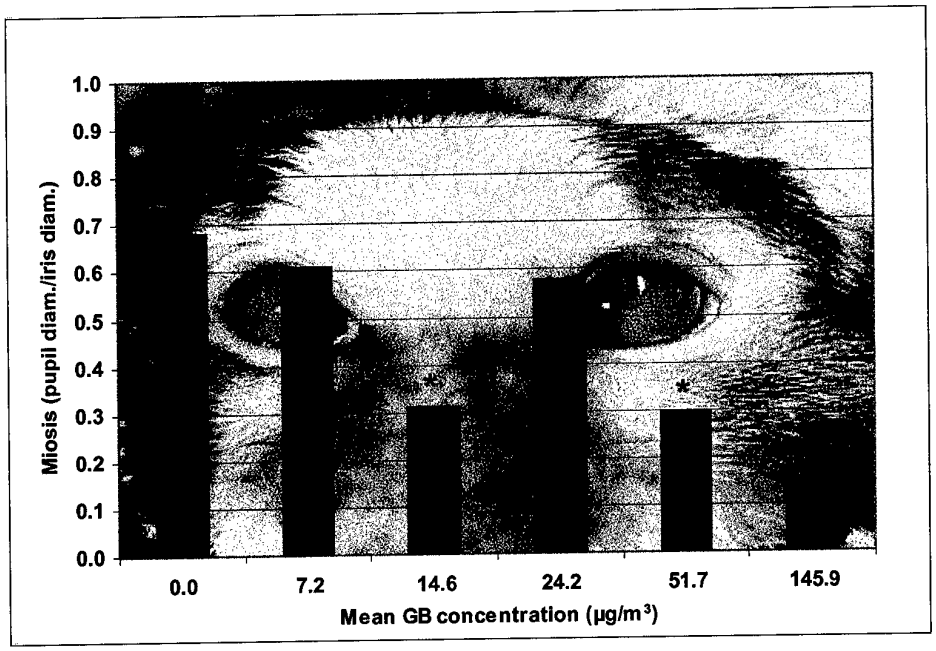

Fig 44. Miosis of 5 restrained conscious marmosets provided with Alzet pumps containing pyridostigmine, after a 5 h exposure to either air (0.0, control, mean ± SEM), or after a 5 h exposure to various mean concentrations of GB (one animal per concentration). (, significantly different from control, $p < 0.05$).*

Table 23. Exposure times needed to achieve significant ($p < 0.05$) miosis during exposure to the various mean GB concentrations and the corresponding C.t values. The concentrations given in the table are the actual mean concentrations between $t = 0$ and the time point at which miosis became significant different from the control value. Nm = not measured.

Pyridostigmine-pretreatment		
Mean (± sem) *conc.of GB exposure (ug/m^3)	Time (min) to significant miosis ($p<0.05$) (min)	C.t ($mg.min.m^{-3}$)
7.2 ± 0.2	225	1.61
15 ± 0.4	111	1.67
23.9	Nm**	-
41.8 ± 1.7	114	4.74
146.9 ± 1.1	27	3.96
	Mean ± sem	3.0 ± 0.8

**Time-based average of vapor concentrations measured at 2-5 min intervals*

*** This animal kept its eyes closed.*

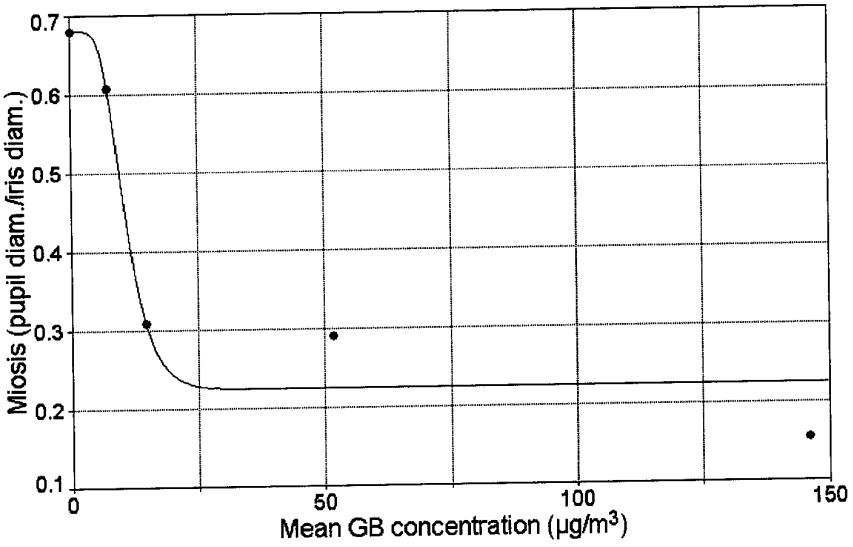

Fig 45. Relationship between the degree of miosis at the end of a 5 h exposure to various mean concentrations of GB, and the mean exposure concentrations of GB, for pyridostigmine-pretreated marmosets. The points to be fitted were taken from Fig 44.

2. EEG

Vehicle-pretreatment

The analysis of the online registered EEG epochs from vehicle-pretreated marmosets (n = 5) during a 5 h exposure to air is demonstrated in Fig 46. The averaged amounts of energy per band (d_1, d_2, t_1 etc.) did not change significantly between t = 0 and t = 300 min of exposure.

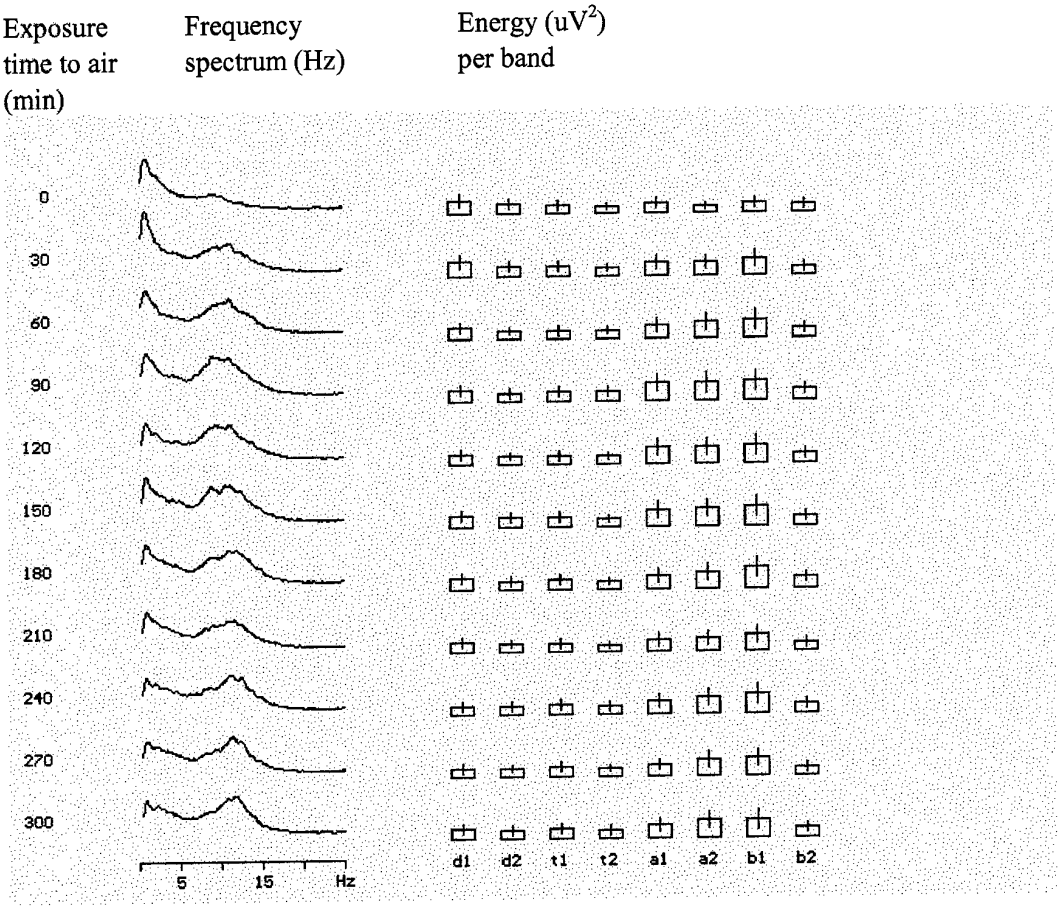

Fig 46. EEG analysis of marmosets (n = 5) provided with Alzet pumps containing vehicle and exposed to air for 5 h. Indicated are the exposure time intervals (min) at which the EEG-analysis was carried out, the averaged frequency spectrum (Hz) and the averaged energy (μV^2) per EEG-band.

These averaged amounts of energy per EEG band of vehicle-pretreated and air-exposed animals were compared to the amounts of energy of the corresponding EEG bands of vehicle-pretreated plus GB-exposed animals (7.3, 14.6, 21.8, 49.7 or 137.7 $\mu g/m^3$, one animal per concentration).

In Table 22 only the EEG changes are given which appeared to be significantly different ($p < 0.05$) from that in air-exposed animals. For all signifcant changes given in Table 22, the *LOAEL* (C.t) values were calculated using the actual GB concentrations (not shown) instead of the indicated mean concentrations in the table. These *LOAEL* values are given in Fig 47.

Table 22. Statistically analyzed differences between EEG-bands from vehicle-pretreated and GB-exposed (7.3, 14.6, 21.8, 49.7, or 137.7 µg/m³ GB, one animal per concentration) marmosets, and the corresponding EEG-bands from vehicle-pretreated and air-exposed (n = 6) animals. Indicated are the EEG-bands which are significantly different (p<0.05) from the corresponding bands in air exposed animals.

Mean GB conc. (µg/m³)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.3 ± 0.1	d_2 b_2	b_2	d_2 t_1 b_2	t_1	d_2 t_1 t_2	t_1 t_2 b_2	t_1 t_2 b_2	t_1 t_2 b_2	t_1	t_1
14.6 ± 0.2								b_2		t_1 b_2
21.8 ± 0.4	a_1	t_1 t_2 a_1	t_1 t_2 a_1		t_2 a_1	t_2 a_1	t_1 t_2 a_1	t_2 a_1 b_2	t_2 b_2	t_2 b_2
49.7 ± 0.6						b_2		b_2		
137.7 ± 1.7		d_1 t_1 t_2 b_1	t_1	t_1 t_2	d_1 t_1 t_2	t_1 t_2 b_2	t_1 t_2 a_1 a_2			b_2

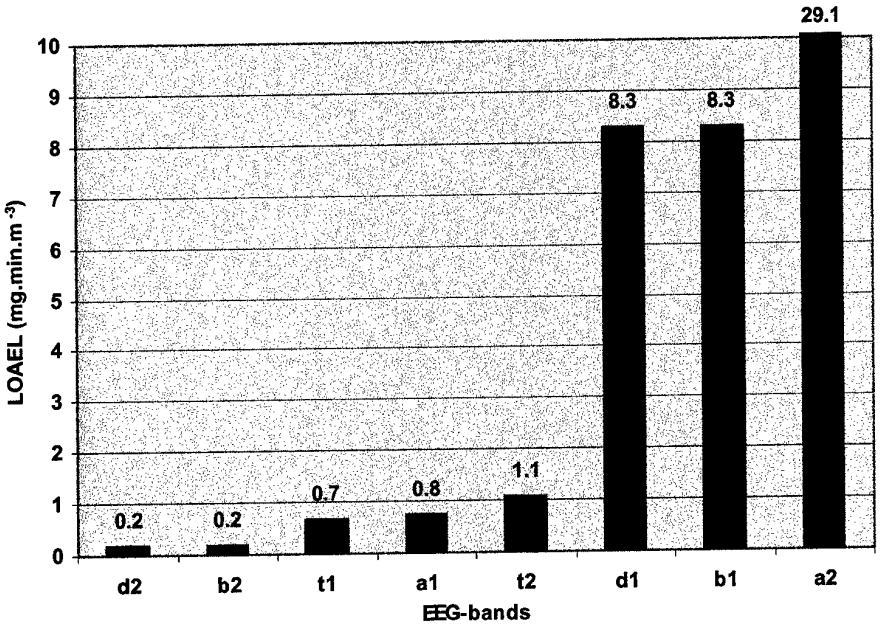

Fig 47. EEG-bands (horizontal axis) of vehicle-pretreated GB-exposed marmosets which became first significantly (p < 0.05) different from the corresponding bands in air exposed animals, and the calculated corresponding LOAEL levels (vertical axis).

It appeared that the d_2 and b_2 EEG-bands were most sensitive to GB exposure, whereas the d_1, b_1 and a_2 bands were least sensitive. The lowest C.t-value established in this way was 0.2 mg.min.m^{-3}, representing the LOAEL for the first emerging EEG changes in vehicle-pretreated and GB-exposed marmosets.

Pyridostigmine-pretreatment

The analysis of the online registered EEG epochs from pyridostigmine-pretreated marmosets (n = 5) during a 5 h exposure to air is demonstrated in Fig 48. The averaged amounts of energy per band (d_1, d_2, t_1 etc.) did not change significantly between t = 0 and t = 300 min of exposure.

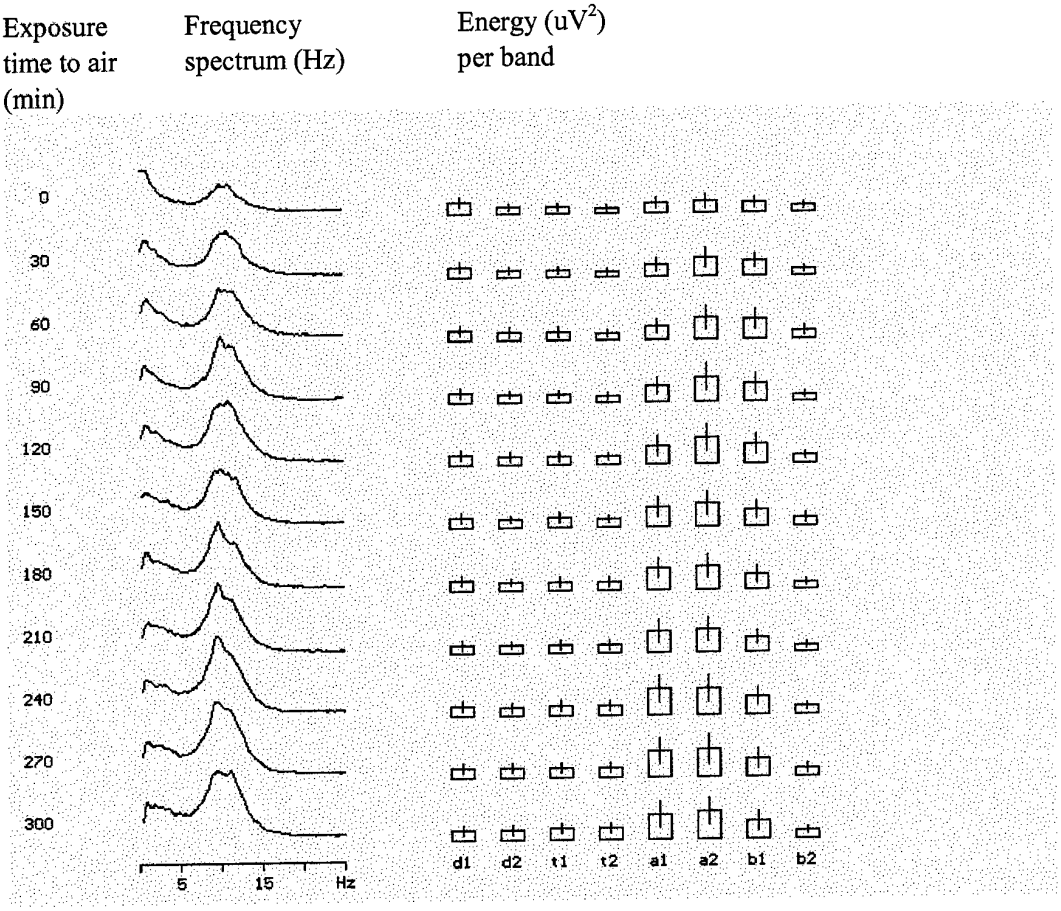

Fig 48. EEG analysis of marmosets (n = 5) provided with Alzet pumps containing pyridostigmine (0.04 mg/kg/h) and exposed to air for 5 h. Indicated are the exposure time intervals at which the EEG-analysis was carried out, the averaged frequency spectrum and the averaged energy (uV^2) *per EEG-band per time interval.*

These averaged amounts of energy per EEG band of pyridostigmine-pretreated and air-exposed animals were compared with the amounts of energy of the corresponding EEG bands of pyridostigmine-pretreated and GB-exposed animals (7.2, 14.6, 24.2, 51.7 or 145.9 $\mu g/m^3$, one animal per concentration). In Table 23 only the EEG changes which appeared to be significantly different ($p < 0.05$) from that in air-exposed animals are given. For all signifcant changes given in Table 23, the LOAEL (C.t) values were calculated using the actual GB concentrations (not shown) instead of the indicated mean concentrations in the table. These LOAEL values are given in Fig 49. It appeared that the t_1 and d_2 EEG-bands were most sensitive to GB exposure, whereas the b_1 band was least sensitive. The lowest C.t-value established in this way was 0.1 $mg.min.m^{-3}$, representing the *LOAEL* for the first emerging EEG changes in pyridostigmine-pretreated and GB-exposed marmosets.

Table 23. Statistically analyzed differences between EEG-bands from vehicle-pretreated and GB-exposed (7.2, 14.6, 24.2, 51.7, or 145.9 μg/m³ GB, one animal per concentration) marmosets, and the corresponding EEG-bands from vehicle-pretreated and air-exposed (n = 6) animals. Indicated are the EEG-bands which are significantly different (p<0.05) from the corresponding bands in air exposed animals.

Mean (± SEM)* GB conc. (μg/m³)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.2 ± 0.1	t_1			b_2			b_2		b_2	
14.6 ± 0.2	d_2 t_1	d_2	d_2 t_1	d_2 t_1 t_2	d_1 d_2 t_1 t_2 a_1 a_2	d_1 d_2 t_1 t_2 a_1 a_2	d_1 d_2 t_1 t_2 a_1 a_2	d_2 t_1 t_2 a_2	d_2 t_1 t_2	d_1 d_2 t_1 a_1 a_2
24.2 ± 0.9	d_2	d_2 a_2	d_1 d_2 t_1 t_2	d_2 t_1 a_2	d_2 a_2	a_2	d_2 a_2	d_2 t_1 a_2	a_2	a_2 b_1
51.7 ± 1.2										
145.9 ± 1.3	t_1									

**Time-based average of vapor concentrations measured at 2-5 min intervals*

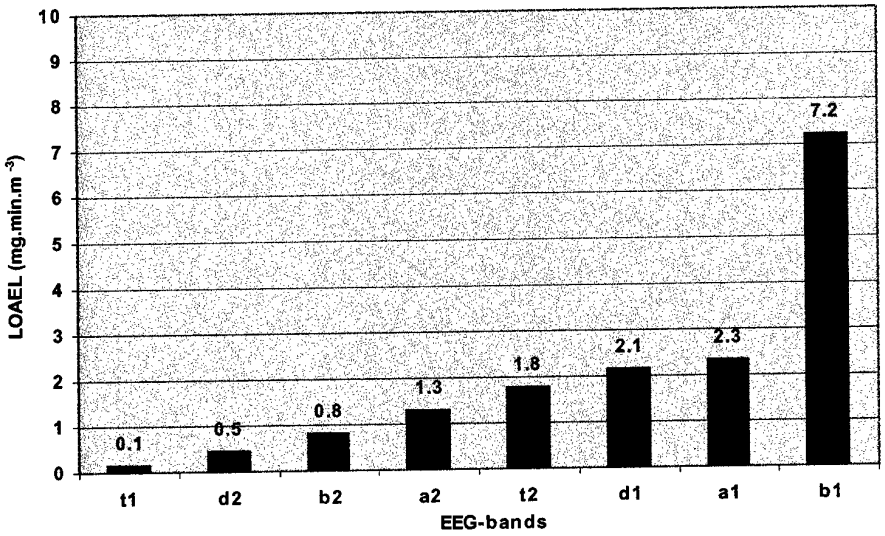

Fig 49. EEG-bands of pyridostigmine-pretreated GB-exposed marmosets which became first significantly (p < 0.05) different from the corresponding bands in air exposed animals (horizontal axis), and the calculated corresponding LOAEL levels (vertical axis).

Fig 50 shows the differences in *LOAEL* (C.t) levels between vehicle or pyridostigmine-pretreated GB-exposed animals regarding the first changing EEG-bands. The d_1, t_1 and a_2 bands in pyridostigmine-pretreated animals are more sensitive for GB than the corresponding bands in vehicle-pretreated animals. On the other hand, the a_1 and b_2 bands in vehicle-pretreated animals are more sensitive for GB than the corresponding bands in pyridostigmine-pretreated animals.

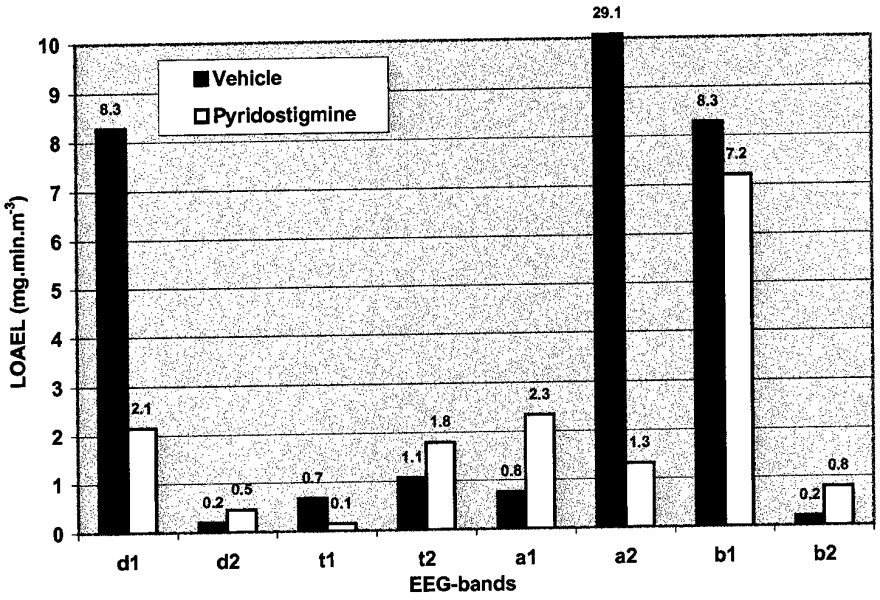

Fig 50. Comparison of EEG-bands (horizontal axis) of vehicle-pretreated (blue) or pyridostigmine-pretreated (white) GB-exposed marmosets which became first significantly ($p < 0.05$) different from the corresponding bands in air exposed animals, and of the calculated corresponding LOAEL levels (vertical axis).

3. *Visual-evoked response (VER)*

Vehicle-pretreatment

The averaged VER signals of 5 vehicle-pretreated marmosets (M11-M15) for each exposure time interval are shown in Fig 51. In this figure the mean ± SEM of the VER latencies t_2 and t_3 have also been demonstrated (compare Fig 5, Materials & Methods) This is different from the way of presentation of the guinea pig data (see page 13), although for both species the same procedure for analyzing the data was used. As the VER-signal of the marmoset differs from that of the guinea pig, only t_2 and t_3 were taken into account for marmosets (Fig 51).

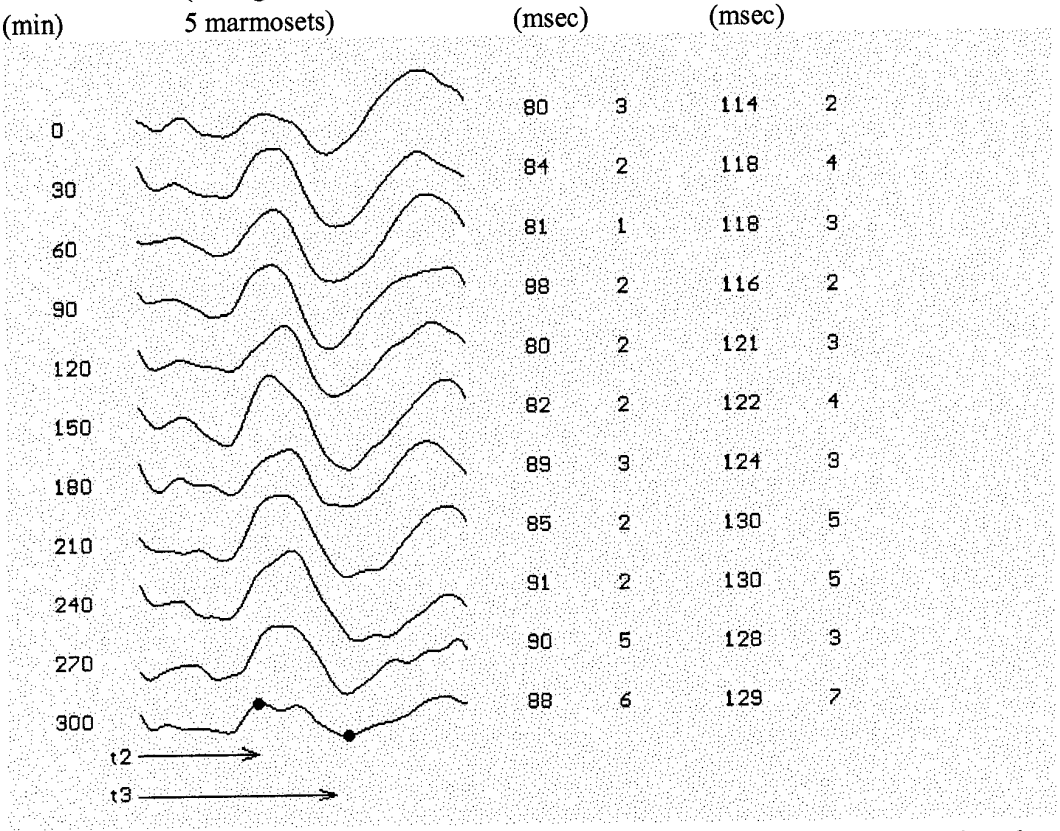

Fig 51. VER analysis of 5 marmosets (M11-M15) provided with Alzet pumps containing vehicle and exposed to air for 5 h. Indicated are the exposure time intervals (min) at which the VER analysis was carried out, the averaged VER-signal of the animals, and the mean (± SEM) VER latencies t_2 and t_3 calculated from the individual curves.

The same animals (M11-M15) were exposed to GB (one animal per concentration) two days after the exposure to air.

The corresponding VER latencies t_2 and t_3 obtained from vehicle-pretreated marmosets (M11-M15) during a 5 h exposure to various concentrations of GB are shown in Table 24.

Table 24. VER latencies t_2 and t_3 obtained from vehicle-pretreated marmosets (M11 –M15) during a 5 h exposure to various mean concentrations of GB vapor in air (7.3, 14.6, 21.8, 49.7, or 137.7 µg/m³, one animal per concentration). (-, not determined).

Exposure time to GB (min)	M11		M12		M13		M14		M15	
	137.7 µg/m³ GB		49.7 µg/m³ GB		21.8 µg/m³ GB		14.6 µg/m³ GB		7.3 µg/m³ GB	
	t2	t3	t2	t3	t2	t3	t2	t3	t2	t3
0	105	121	83	112	86	120	91	108	75	115
30	94	105	75	106	80	118	93	108	85	122
60	86	125	71	106	86	113	92	106	75	121
90	104	126	77	119	81	117	91	110	89	125
120	104	127	79	119	82	115	76	107	95	137
150	123	154	-	-	85	114	81	109	88	132
180	133	160	81	133	91	130	84	115	92	133
210	126	152	82	119	91	136	92	138	91	129
240	127	142	78	121	99	148	91	127	93	134
270	94	111	78	115	95	147	97	141	92	136
300	95	111	79	115	76	124	84	113	93	128

Significant ($p < 0.05$) differences between VER latencies t_2 and t_3 (as shown in Fig 51) and the corresponding latencies during exposure to various concentrations of GB (as shown in Table 24), are demonstrated in Table 25. It should be emphasized that before statistical analysis the VER-latency values for t_2 and t_3 (Fig 51 and Table 24) were standardized as follows. Let A and B be the VER-latency t_2 at t = 0 and t = 30 min, respectively. Then the standardized VER-latency t_2 at t = 30 min was set to A/B + B/A. The same calculations were done at t = 60 and 90 min etc. The student t-test was used to compare the standardized VER-latencies per animal exposed to GB (Table 24) with the averaged standardized VER-latencies at the corresponding time intervals from the six air-exposed animals (Fig 51). At t = 180 min during exposure to a mean GB concentration of 137.7 µg/m³, t_2 was significantly different from the corresponding t_2 in air-exposed animals. Since the actual mean GB concentration between t = 0 and t = 180 min was 139 µg/m³, the *LOAEL* for the first VER-changes (t_2) in vehicle-pretreated marmosets was calculated to be 25 mg.min.m^{-3}.

Table 25. Results of statistically analyzed differences between the VER-latencies t_2 and t_2 obtained on-line from 5 restrained conscious and vehicle-pretreated marmosets (M11-M15) during a 5 h exposure period to either air or various concentrations of GB vapor in air (7.3, 14.6, 21.8, 49.7, or 137.7 $\mu g/m^3$, one animal per concentration). Indicated are the latencies which are significantly different ($p < 0.05$) from the corresponding latencies in air exposed animals. Note that at t = 180 min during exposure to a mean GB concentration of 137.7 $\mu g/m^3$, t_2 was significantly different from the corresponding t_2 in GB-exposed animals.

Mean (± SEM *conc. of GB ($\mu g/m^3$)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.3 ± 0.1										
14.6 ± 0.2										
21.8 ± 0.4										
49.7 ± 0.6										
137.7 ± 1.7						t_2				

**Time-based average of vapor concentrations measured at 2-5 min intervals*

Pyridostigmine-pretreatment

The averaged VER signals of 5 pyridostigmine-pretreated marmosets (M16-M20) for each exposure time interval, and the mean VER latencies t_2 and t_3 calculated from the individual curves are demonstrated in Fig 52.

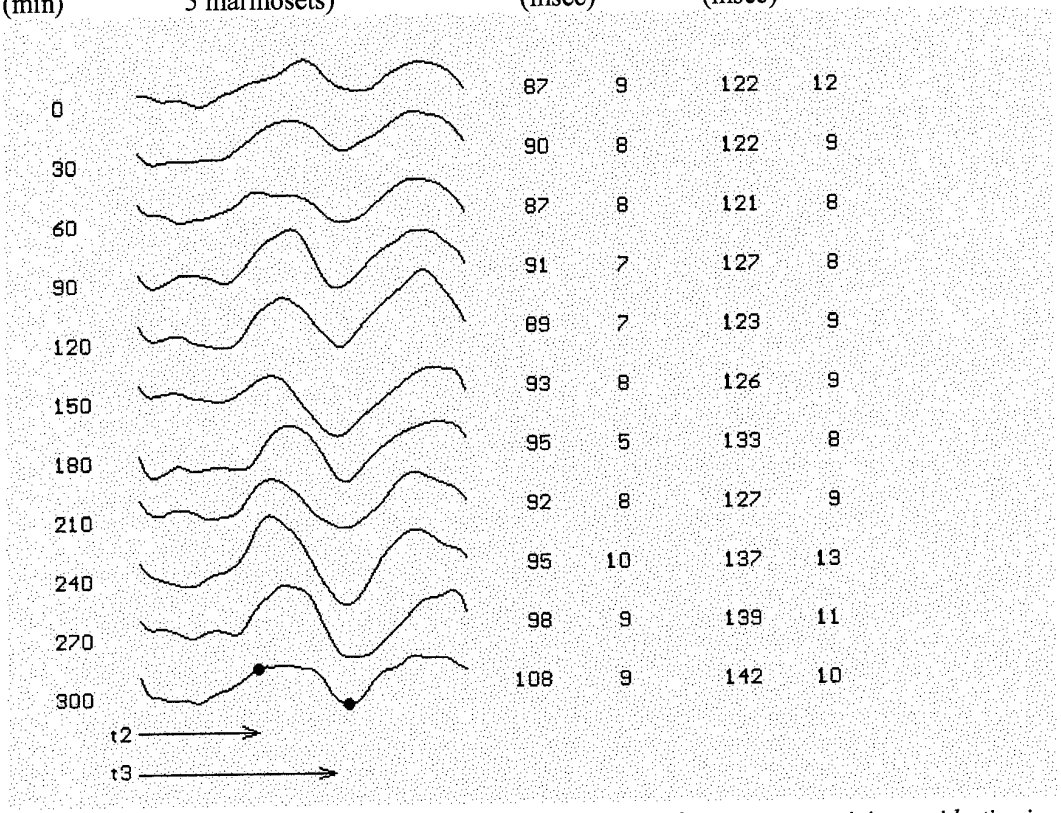

Fig 52. VER analysis of 5 marmosets (M16-M20) provided with Alzet pumps containing pyridostigmine and exposed to air for 5 h. Indicated are the exposure time intervals (min) at which the VER analysis was carried out, the averaged VER-signal from 5 animals, and the mean (± SEM) VER-latencies t_2 and t_3 calculated from the individual curves.

The same animals (M16-M20) were exposed to GB (one animal per concentration) two days after the exposure to air.

The corresponding VER latencies t_2 and t_3 obtained from vehicle-pretreated marmosets (M16-M20) during a 5 h exposure to various concentrations of GB are shown in Table 26.

Table 26. VER latencies t_2 and t_3 obtained from pyridostigmine-pretreated marmosets (M16 –M20) during a 5 h exposure to various mean concentrations of GB vapor in air (7.2, 14.6, 24.2 ,51.7, 145.9 $\mu g/m^3$, one animal per concentration).

Exposure time to GB (min)	M16		M17		M18		M19		M20	
	145.9 µg/m³ GB		51.7 µg/m³ GB		24.2 µg/m³ GB		14.6 µg/m³ GB		7.2 µg/m³ GB	
	t2	t3	t2	t3	t2	t3	t2	t3	t2	t3
0	103	137	88	120	77	96	90	116	82	120
30	81	117	97	127	78	88	84	113	82	120
60	87	126	92	129	72	91	86	113	80	123
90	79	129	91	134	83	107	110	82	90	119
120	94	125	102	146	83	101	74	114	95	128
150	94	130	95	134	74	92	88	120	86	128
180	101	125	104	142	73	95	88	121	89	120
210	93	122	105	133	74	98	90	126	96	123
240	97	126	97	128	82	95	90	124	91	136
270	87	117	83	126	78	92	91	127	84	133
300	88	117	86	142	82	104	92	127	88	141

Significant ($p < 0.05$) differences between VER latencies t_2 and t_3 (as shown in Fig 52) (air exposure) and the corresponding latencies during exposure to various concentrations of GB (as shown in Table 26), are given in Table 27. It should be emphasized that before statistical analysis the VER-latency values for t_2 and t_3 (Fig 52 and Table 26) were standardized as follows. Let A and B be the VER-latency t_2 at t = 0 and t = 30 min, respectively. Then the standardized VER-latency t_2 at t = 30 min was set to A/B + B/A. The same calculations were done for at t = 60 and 90 min etc. The student t-test was used to compare the standardized VER-latencies per animal exposed to GB (Table 26) with the averaged standardized VER-latencies at the corresponding time intervals from the six air-exposed animals (Fig 52). The significant VER-change (t_2) at t = 30 min during exposure to a mean GB concentration of 145.9 $\mu g/m^3$, resulted in the lowest *LOAEL* (C.t) value regarding VER changes in pyridostigmine-pretreated marmosets: 4.4 $mg.min.m^{-3}$.

Table 27. Results of statistically analyzed differences between the VER-latencies t_2 and t_3 obtained on-line from 5 restrained conscious and pyridostigmine-pretreated marmosets during a 5 h exposure period to either air, or to various mean concentrations of GB vapor in air (7.2, 14.6, 24.2, 51.7, or 145.9 $\mu g/m^3$, one animal per concentration). Indicated are the latencies which are significantly different ($t > 2$; $p < 0.05$) from the corresponding latencies in air exposed animals.

Mean (± SEM) *conc. of GB ($\mu g/m^3$)	Exposure time (min)									
	30	60	90	120	150	180	210	240	270	300
7.2 ± 1.0										
14.6 ± 0.2										
24.2 ± 0.9								t_2		
51.7 ± 1.2							t_2			
145.9 ± 1.3	t_2	t_2	t_2							

**Time-based average of vapor concentrations measured at 2-5 min intervals*

4. Startle-response

Vehicle-pretreatment

The startle-responses of individual vehicle-pretreated marmosets (M11, M12 etc) 1.5 h after the end of a 5 h exposure to air under restrained conditions (stress), did not differ significantly ($p > 0.05$) from the averaged (mean ± SEM) startle-response measured in the same animals before restrainment and exposure to air (M11-M15, Fig 53). The mean (± SEM) of M11, 12, 13, 14 and 15 in Fig 53 was then calculated and given in Fig 54. There was no significant difference between the mean given in Fig 53 (unrestrained) and that given in Fig 54 (restrained).

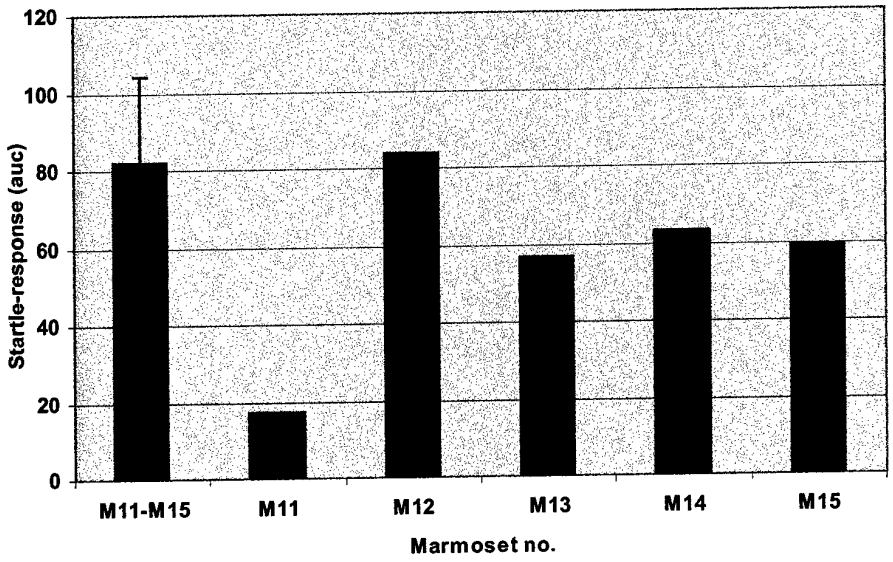

Fig 53. Startle response of 5 vehicle-pretreated marmosets (M11-M15) before entering the exposure chamber for exposure to air under restrained conditions (mean ± SEM), compared to their individual responses (M1, M12 etc) 1.5 h after the end of a 5 h exposure to air.

The startle-response of individual vehicle-pretreated marmosets at the end of a 5 h whole-body exposure to a mean concentration of either 7.3, 14.6, 21.8, 49.7 or 137.7 $\mu g/m^3$ GB vapor in air, is given in Fig 54. A 5 h exposure to a mean concentration of 21.8 $\mu g/m^3$, resulted in a significant ($p < 0.05$) increase in response. This would result in a *LOAEL* (C.t-value) of 6.5 $mg.min.m^{-3}$ for the startle-response in vehicle-pretreated marmosets. Although we have no explanation for the observation that there was only a significant effect at 21.8 $\mu g/m^3$, the concentration range tested may be considered as a threshold range for emerging effects on startle-response in vehicle-pretreated marmosets.

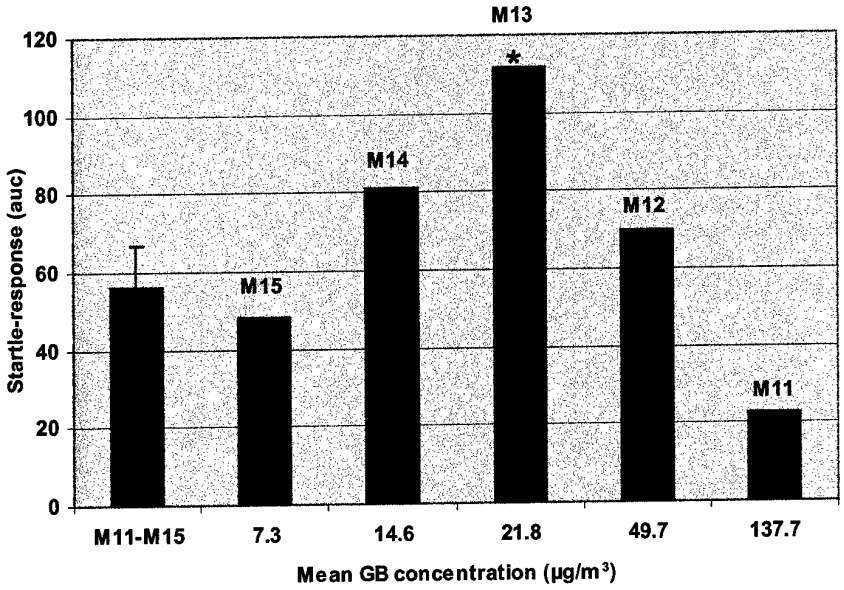

Fig 54. Startle response of 5 vehicle-pretreated marmosets (M11-M15) 1.5 h after the end of a 5 h exposure to air (mean ± SEM of the individualvalues scored by M11, 12, 13, 14 and 15 taken from Fig 53), compared to their individual responses 1.5 h after the end of a 5 h exposure to various mean concentrations of GB (7.3, 14.6, 21.8, 49.7, 137.7 µg.min.m^{-3}, one animal per concentration). (, significantly different from the mean control value, $p < 0.05$).*

Pyridostigmine-pretreatment

The startle-responses of 2 out of 5 individual pyridostigmine-pretreated marmosets 1.5 h after the end of a 5 h exposure to air under restrained conditions (stress), differed significantly ($p < 0.05$) from the averaged (mean ± SEM) startle-response measured in the same pyridostigmine-pretreated animals before restrainment and exposure to air (M16-M20, Fig 55). The mean (± SEM) of M16, 17, 18, 19 and 20 in Fig 55 was then calculated and given in Fig 56. There was a significant ($p < 0.05$) difference between the mean given in Fig 55 (unrestrained) and that given in Fig 56 (restrained), indicating that restrainment (stress) of pyridostigmine-pretreated animals decreases the startle-response (see also Fig 57).

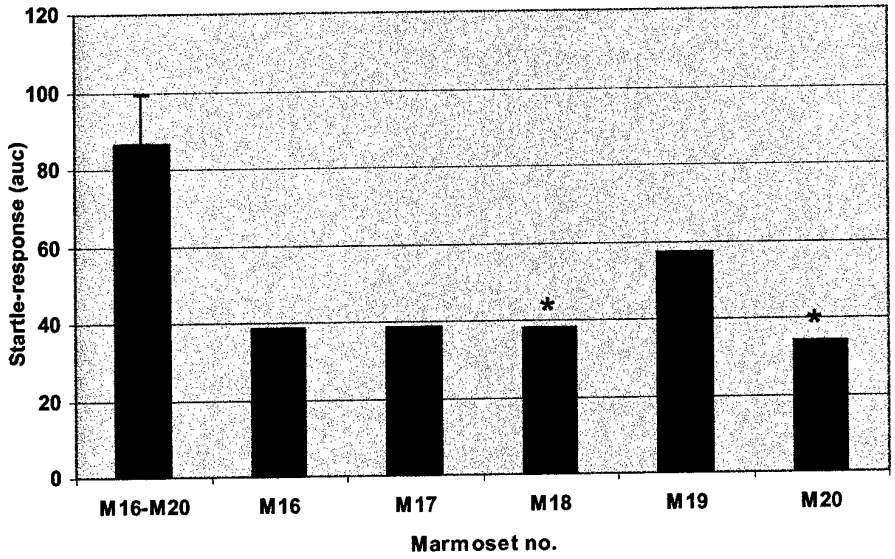

Fig 55. Startle response of 5 pyridostigmine- pretreated marmosets (M16-M20) before entering the exposure chamber under restrained conditions (stress) (mean ± SEM), compared to their individual responses 1.5 h after the end of a 5 h exposure to air. (, significantly different from the mean control value, $p < 0.05$).*

The startle-response of the same pyridostigmine-pretreated animals (M16-M20) at the end of a 5 h exposure to a mean concentration of either 7.2, 14.6, 24.2, 51.7, or 145.9 $\mu g/m^3$ GB, differed significantly ($p < 0.05$) only at a concentration of 14.6 $\mu g/m^3$ GB from the averaged startle-response measured in 5 pyridostigmine-pretreated animals at the end of a 5 h exposure to air (Fig 56). This would result in a *LOAEL* (C.t-value) of 4.4 $mg.min.m^{-3}$ for the startle-response in pyridostigmine-pretreated marmosets.

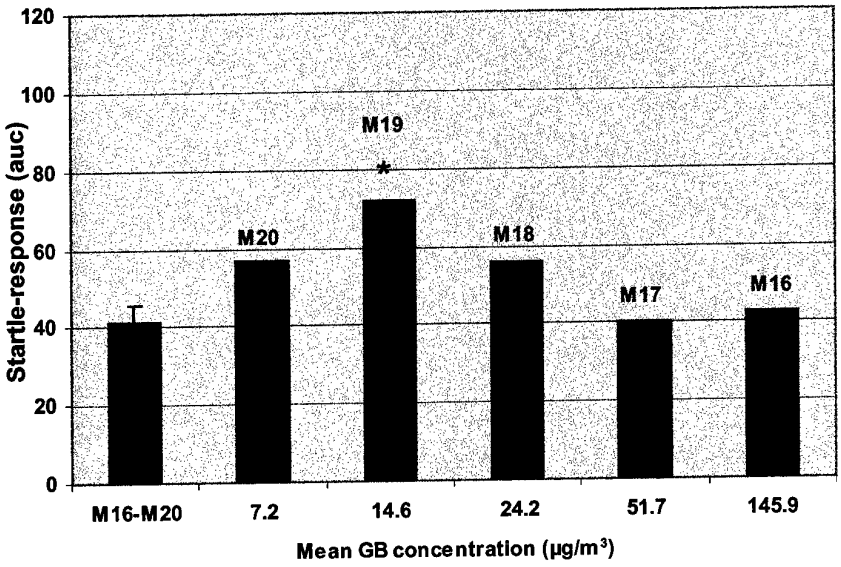

Fig 56. Startle response of 5 pyridostigmine-pretreated marmosets (M16-M20) 1.5 h after the end of a 5 h exposure to air (mean ± SEM of the individual values scored by M16, 17, 18, 19 and 2 taken from Fig 55), compared to their individual responses 1.5 h after the end of a 5 h exposure to various mean concentrations of GB (7.2, 14.6, 24.2, 51.7, 145.9 µg.min.m^{-3}, one animal per concentration). (, significantly different from the mean control value, $p < 0.05$).*

The averaged startle-response of 5 vehicle-pretreated animals (M11-M15) 1.5 h after a 5 h exposure to air (under restrained conditions), did not differ significantly ($p > 0.05$) from that before or 24 h after the exposure to air (Fig 57, left panel). In contrast, the averaged startle-response of 5 pyridostigmine-pretreated animals (M16 M20) 1.5 h after a 5 h exposure to air, did differ significantly ($p < 0.05$) from that before or 24 h after the exposure to air (Fig 57, right panel).

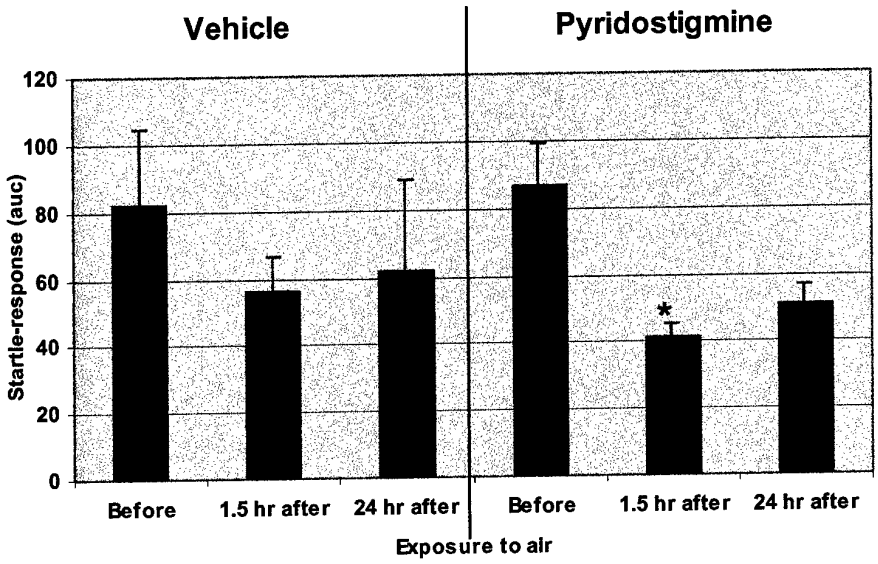

Fig 57. Averaged (mean ± SEM) startle response of 5 vehicle-pretreated (M11-M15) (left panel) and 5 pyridostigmine-pretreated (right panel) marmosets (M16 –M20) before entering the exposure chamber for exposure to air (under restrained (stress) conditions, control), 1.5 h and 24 h after a 5 h exposure to air.(, significantly different from the startle response measured before exposure to air, $p < 0.05$).*

5. Bungalow-test

Vehicle-pretreatment

The bungalow-test responses of individual vehicle-pretreated marmosets (M11, M12 etc) 1.5 h after the end of a 5 h exposure to air under restrained conditions (stress), did not differ significantly from the averaged (mean ± SEM) bungalow-test response measured in the same animals before restrainment and exposure to air (Fig 58). The mean (± SEM) of the individual scores by M11, 12, 13, 14 and 15 taken from Fig 58 (after restrainment), is given Fig 59. The mean value given in Fig 58 did not differ significantly ($p < 0.05$) from that given in Fig 59, indicating that performance of vehicle-pretreated animals is not decreased after restrainment (see also Fig 62).

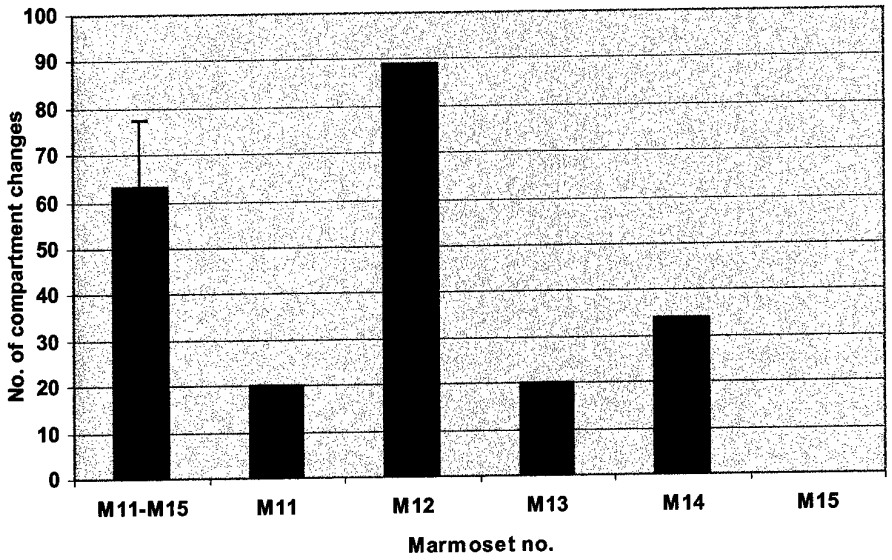

Fig 58. Bungalow-test response (number of compartment changes) of 5 vehicle-pretreated marmosets (M11-M15) before entering the exposure chamber (mean ± SEM), compared to their individual responses (M11, M12 etc)1.5 h after the end of a 5 h exposure to air under restrained conditions (stress).

The bungalow-test response of individual vehicle-pretreated marmosets at the end of a 5 h whole-body exposure to a mean concentration of either 7.3, 14.6, 21.8, 49.7 or 137.7 $\mu g/m^3$ GB vapor in air, is given in Fig 59. Exposure to a mean concentration of 49.7 $\mu g/m^3$, was the only exposure level that resulted in a significant ($p < 0.05$) increase in response. This would result in a *LOAEL* (C.t-value) of 14.9 $mg.min.m^{-3}$ for the bungalow-test response in vehicle-pretreated marmosets.

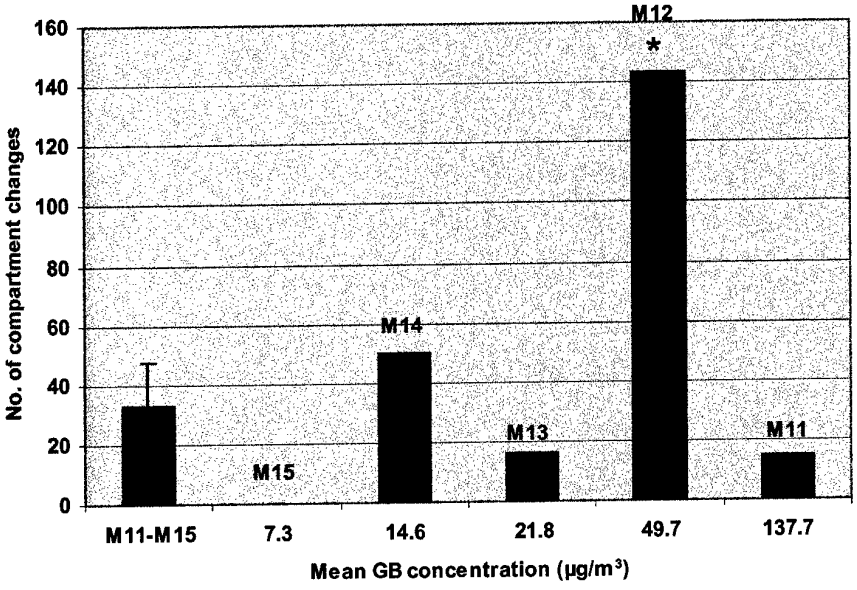

Fig 59. Bungalow-test response (number of compartment changes) of 5 vehicle-pretreated marmosets (M11-M15) 1.5 h after the end of a 5 h exposure to air (mean ± SEM of the individual values scored by M11, 12, 13, 14 and 15, see Fig 58), compared to their individual responses 1.5 h after the end of a 5 h exposure to various mean concentrations of GB (7.3, 14.6, 21.8, 49.7, 137.7 µg.min.m^{-3}, one animal per concentration). (, significant increase in response, as compared to the mean control value, $p < 0.05$).*

Pyridostigmine-pretreatment

The bungalow-test responses of 3 (M16, M18 and M19) out of 5 individual pyridostigmine-pretreated marmosets (M16-M20) 1.5 h after the end of a 5 h exposure to air under restrained conditions (stress), differed significantly ($p < 0.05$) from the averaged (mean ± SEM) bungalow-test response measured in the same animals before restrainment and exposure to air (Fig 60). The mean (± SEM) of the individual scores by M16, 17, 18, 19 and 20 taken from Fig 60 (after restrainment), is given Fig 61. The mean value given in Fig 60 differs significantly ($p < 0.05$) from that given in Fig 61, indicating that performance of pyridostigmine-pretreated animals is decreased after restrainment (see also Fig 62).

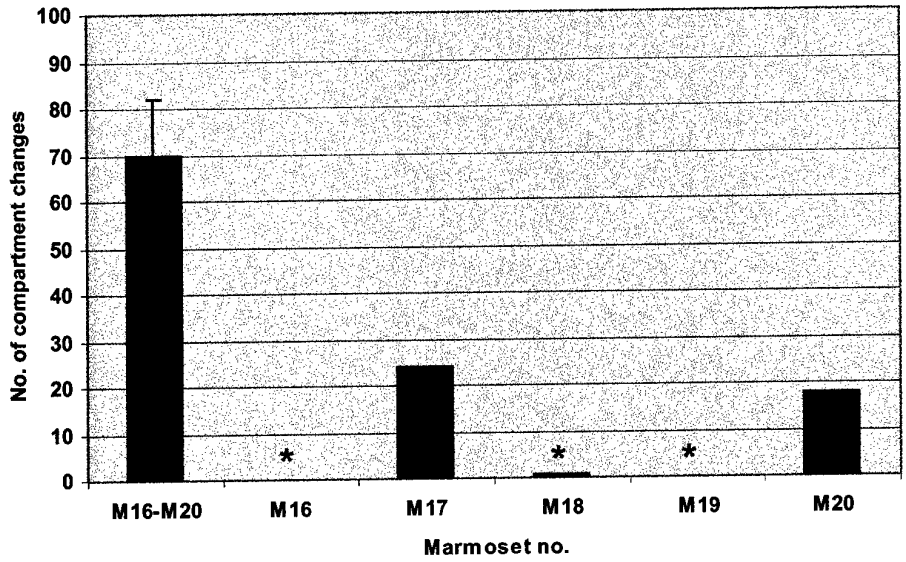

*Fig 60. Bungalow-test response (number of compartment changes) of 5 pyridostigmine- pretreated marmosets (M16-M20) before entering the exposure chamber (mean ± SEM), compared to their individual responses (M16, M17 etc) 1.5 h after the end of a 5 h exposure to air.(*, significant decrease in response compared to the mean control value, $p < 0.05$).*

The bungalow-test response of the same pyridostigmine-pretreated animals at the end of a 5 h exposure to a mean concentration of either 7.2, 14.6, 24.2, 51.7, or 145.9 $\mu g/m^3$ GB (one animal per concentration), differed significantly ($p < 0.05$) at concentrations of 24.2 and 51.7 $\mu g/m^3$ GB from the averaged response measured in the same pyridostigmine-pretreated animals at the end of a 5 h exposure to air (Fig 61). This would result in a *LOAEL* (C.t-value) of 7.2 $mg.min.m^{-3}$ for the bungalow-test response in pyridostigmine-pretreated animals for the exposure to a concentration of 24.2 $\mu g/m^3$.

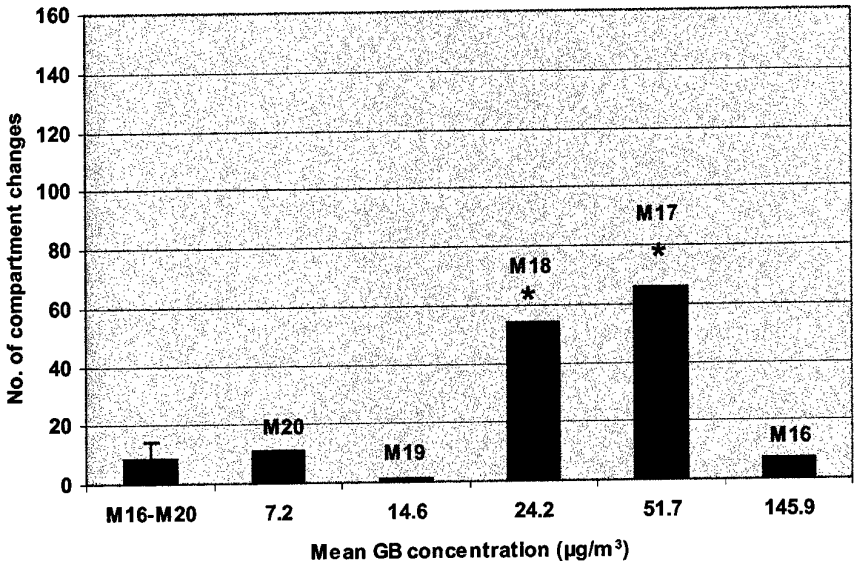

Fig 61. Averaged (mean ± SEM calculated from the individual values of M16, 17, 18, 19, 20 taken from Fig 60) bungalow-test response (number of compartment changes) of 5 pyridostigmine-pretreated marmosets 1.5 h after the end of a 5 h exposure to air, compared to their individual responses 1.5 h after the end of a 5 h exposure to different mean concentrations of GB (7.2, 14.6, 24.2, 51.7, 145.9 µg.min.m^{-3}, one animal per concentration). (, significant increases in response as compared to the mean control value, $p < 0.05$).*

The averaged bungalow-test response of 5 vehicle-pretreated animals (M11-M15) 1.5 h after a 5 h exposure to air under restrained conditions, did not differ significantly ($p > 0.05$) from that before or 24 h after the exposure to air (Fig 62, left panel). In contrast, the averaged startle-response of 5 pyridostigmine-pretreated animals (M16-M20) 1.5 h after a 5 h exposure to air under restrained conditions, did differ significantly ($p < 0.05$) from that before or 24 h after the exposure to air (Fig 62, right panel).

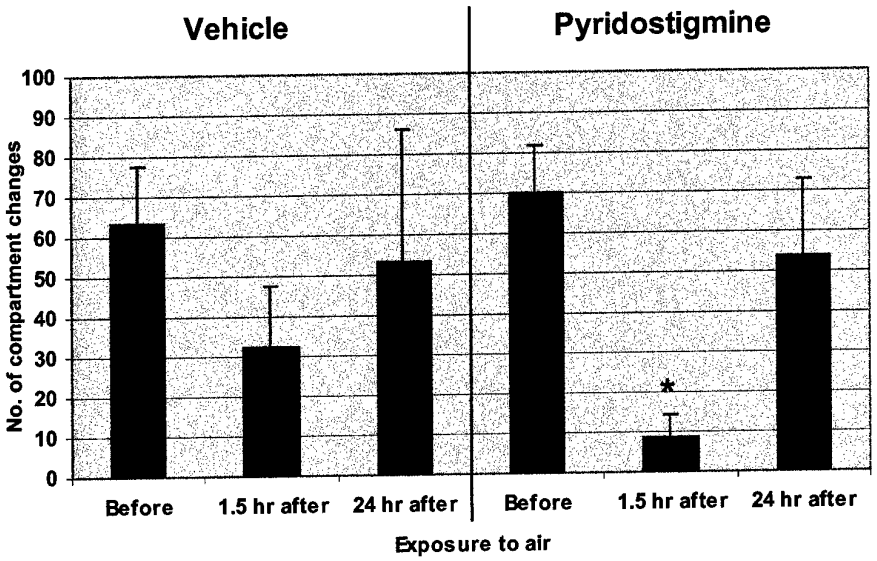

Fig 62. Averaged (mean ± SEM) bungalow-test response (number of compartment changes) of 5 vehicle-pretreated (M11-15) (left panel) and 5 pyridostigmine-pretreated (M16-M20) (right panel) marmosets before entering the exposure chamber to be exposed to air under restrained conditions (stress) (control), 1.5 h and 24 h after a 5 h exposure to air (, significant decrease in response 1.5 h after a 5 h exposure to air, $p < 0.05$).*

Acetylcholinesterase (AChE) activity in blood

Vehicle-pretreatment

A 5 h whole-body exposure of vehicle-pretreated marmosets (M11-M15) to mean GB vapor concentrations of 14.6, 21.8, 49.7 or 137.7 µg/m^3, resulted in significant (*, $p < 0.05$) decreases in AChE-activity in blood, compared to the averaged AChE-activity in blood (97%) from 5 animals 4 days after the insertion (0.0) of the Alzet pump containing vehicle (Fig 63).

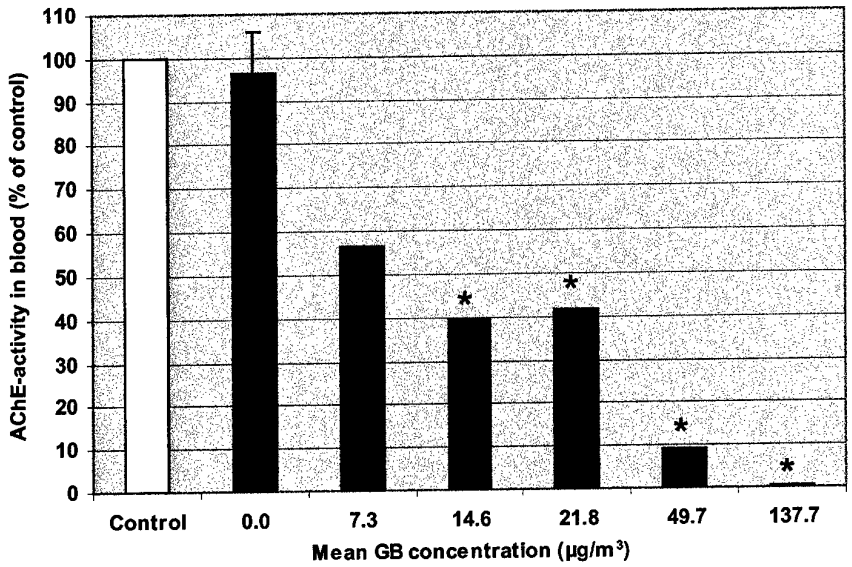

Fig 63. AChE-activity in blood samples taken from 5 marmosets before the insertion of Alzet pumps containing vehicle (set at 100%, control), 4 days after the insertion (0.0) of the pumps (mean ± SEM), and at the end of a 5 h exposure to various concentrations of GB vapor in air (7.3, 14.6, 21.8, 49.7 or 137.7 µg/m^3 GB, one animal per concentration). (, significant decreases in AChE-activity in blood compared to the mean control value, $p < 0.05$).*

Pyridostigmine-pretreatment

The averaged AChE-activity in blood from 5 animals (M16-M20) 4 days after the insertion of Alzet pumps containing pyridostigmine was about 78% (0.0) of their own control values (control, set at 100%), i.e., before implantation of the Alzet pumps containing pyridostigmine (Fig 64). A 5 h exposure to GB vapor concentrations in the range of 7.2 – 145.9 µg/m^3 resulted in significant decreases in blood AChE-activity during 5 h exposures to mean GB concentrations of 14.6, 51.7 or 145.9 µg/m^3. The non-significant difference at a GB concentration of 24.2 µg/m^3 represents the animal which kept its eyes closed during exposure to GB.

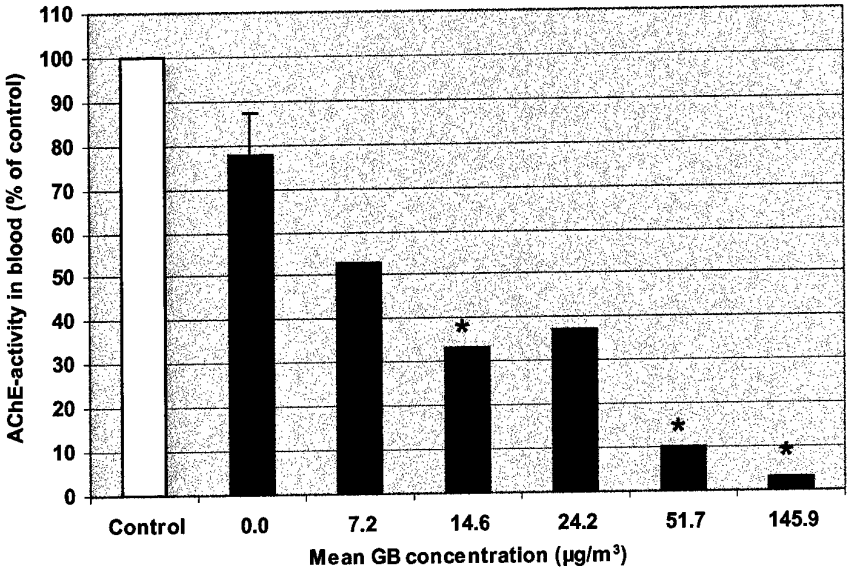

Fig 64. AChE-activity in blood samples taken from 5 marmosets (M16-M20) before the insertion of Alzet pumps containing pyridostigmine (set at 100%, control), 4 days after the insertion of the pumps (0.0) (mean ± SEM), and at the end of a 5 h exposure to various concentrations of GB vapor in air (7.2, 14.6, 24.2, 51.7 or 145.9 µg/m³, one animal per concentration). (, significant decreases in AChE-activity in blood, compared to the mean control value, $p < 0.05$)*

DISCUSSION

The main results obtained in the present study allow us to answer the major questions put forward at the end of the introductory paragraph:
1. What are the *Lowest Observable Effect Levels (LOEL)* for vehicle-pretreated and pyridostigmine-pretreated guinea pigs and marmosets? (see Table 28).
2. What are the *Lowest Observable Adverse Effect Levels (LOAEL)* on performance (miosis, EEG, VER, startle-response, shuttle-box behavior, Bungalow-test) for vehicle-pretreated and for pyridostigmine-pretreated guinea pigs and marmosets? (see Table 29 and 30).
3. Do unexpected adverse effects emerge through the combination of pyridostigmine-pretreatment and GB exposure?

Ad 1.

Table 28. Summary of the estimated LOELs (C.t) for GB exposure of vehicle- or pyridostigmine-pretreated guinea pigs and marmosets during a 5 h exposure to low levels of GB vapor in air.

*LOEL** (mg.min.m^{-3})			
Guinea pig		Marmoset	
Vehicle	Pyrido	Vehicle	Pyrido
0.010 ± 0.002	0.014 ± 0.003	0.04 ± 0.01	0.048 ± 0.002

* It should be emphasized that the *LOEL* (C.t) and miosis (see Table 29) were investigated most thoroughly, whereas changes in the other parameters should be considered indicative (transient) for incapacitation at this moment and require further investigation.

The *LOELs* calculated for marmosets are about 3-fold higher than those calculated for guinea pigs (Table 28). This reflects a technical difference emerging from drawing larger blood sample volumes (500-700 µl) from guinea pigs than from marmosets (200-300 µl), since more blood can be drawn from guinea pigs than from marmosets. As a consequence, fluoride-generated GB could be detected earlier during GB exposure in guinea pigs than in marmosets. After correction for this difference in blood sample volume, the *LOELs* for marmosets are rather similar to those for guinea pigs. If not even the mildest ChE-inhibition in blood should occur, the recommanded occupational exposure limit of 0.1 µg/m^3 for GB should be reconsidered (Mioduszewski et al 1998) (see below).

Ad 2.

Table 29. Estimated LOAEL (C.t) regarding miosis for GB exposure of vehicle- or pyridostigmine-pretreated guinea pigs and marmosets during a 5 h exposure to low levels of GB vapor in air.

	LOAEL (mg.min.m^{-3})			
	Guinea Pig		Marmoset	
Read-out parameter	Vehicle	Pyrido	Vehicle	Pyrido
Miosis	1.8 ± 0.3	1.8 ± 0.5	2.5 ± 0.8	3.0 ± 0.8

The *LOAEL* for miosis was quite similar for both vehicle-pretreated or pyridostigmine-pretreated guinea pigs and marmosets (Table 29). This result should be addressed since at the BioScience Conference at Hunt Valley (2000) a *LOAEL* of 0.2 mg.min.m^{-3} for miosis in pyridostigmine-pretreated guinea pigs was reported. This lower value was obtained by extrapolation from Fig 23 (this report). From this fitted curve the GB-concentration which corresponded with the first statistically significant decrease in pupil size (miosis) was taken for calculating the *LOAEL* at t = 300 min. Since this GB-concentration was far below the

lowest concentration (< 7.5 μg/m^3) to which the animals were exposed, it was decided to prefer the actually delivered concentrations for calculating the *LOAEL*.
The question whether there is loss of vision at these levels of miosis is difficult to answer since it is not clear whether, apart from miosis, the ciliary muscles may also be contracted causing ciliary spasm resulting in accommodation and near-vision. Moreover, unequal miosis in the two eyes, and/or unequal ciliary spasm, would result in spatial distortion and difficulty in judging distance (Koelle, 1994). In addition, the retina, which is rich in cholinergic innervation, is another important locus for the action of systemic GB on vision (Dementi 1994), which could have been the case in our marmosets demonstrating strong inhibition of AChE activity in their blood.

Table 30. Summary of the indicative LOAELs (C.t) regarding EEG/VER, startle-response, shuttle-box behavior and bungalow-test for GB exposure of vehicle- or pyridostigmine-pretreated guinea pigs and marmosets during a 5 h exposure to low levels of GB vapor in air.

	LOAEL (mg.min.m^{-3})			
	Guinea Pig		Marmoset	
Read-out parameters	Vehicle	Pyrido	Vehicle	Pyrido
EEG	0.8	0.4	0.2	0.1
VER	0.8	0.8	25	4.4
Startle-response	>44	15.2	6.5	4.4
Shuttle-box behavior	2.1	60	-	-
Bungalow-test	-	-	14.9	7.2

An interesting, albeit preliminary finding was that during a 5 h lasting exposure to GB, the EEG seemed to be more sensitive for GB than the eye, both in guinea pigs and in marmosets (Table 29 and 30). This requires confirmation by further research, although the EEG is a more difficult parameter to measure and to interpret than miosis. The early EEG changes were reflected by significant ($p < 0.05$) changes in a, b, d and t-bands in both guinea pig and marmosets irrespective of the kind of pretreatment (Fig 25, 27, 47, 49). Whereas in the present study significant changes were found in all EEG-bands during a 5 h exposure to low levels of GB, Duffy and Burchfiel (1980), reporting on effects of GB on EEG in rhesus monkeys, found significant changes in a-, b_2- and d-bands 24 h after i.v. administration of a high dose of GB (5 μg/kg). Although the functional significances of such EEG changes are difficult to understand, changes in a-bands are often associated with visual sensation, changes in b-bands with excitation/cerebral activity, changes in t-bands with emotional stress, and changes in d-bands with serious organic brain damage (Guyton 1981). As presented at the BioScience Review (Hunt Valley, 2000), EEG changes in vehicle-pretreated guinea pigs exposed to GB, were predominantly limited to the a-bands, suggesting that these EEG changes were induced by miosis rather than by direct effects of GB on CNS. This was not the case with marmosets. The EEG changes in the guinea pig are more difficult to understand since no significant inhibition of AChE activity in blood samples was found at the end of the 5 h exposure to GB levels up to 146 μg/m^3 (whereas a similar exposure to GD caused extensive inhibition, see Benschop et al 1998). This is in favor of the suggestion that in guinea pigs EEG changes might be induced by miosis rather than by direct effects of GB on CNS. In contrast, marmosets exposed to similar GB concentrations, demonstrated AChE inhibition levels in blood of 60-95%. Relevant in this respect is a recent report by Pearce et al (1999) in which was concluded that low doses of GB (2.5-3.0 μg/kg, i.m.) that caused blood ChE-inhibition of 36-67% in marmosets, did not cause significant changes in EEG-pattern, nor decrement in cognitive behaviour. In our marmoset experiments, irrespective of the type of

pretreatment, approximately 50% inhibition of AChE-activity in blood was achieved at the end of a 5 h exposure to about 7.5 $\mu g/m^3$ of GB. These animals did not show decrements in performance (bungalow-test) or in startle-response. However, they showed significant ($p < 0.05$) EEG-changes (Fig 47 and 49). Methodological differences between their and our study will contribute to this difference. Although both groups of investigators measured EEG by radiotelemetry, our animals were restrained whilst their monkeys were unrestrained. They investigated cognitive behavior, which is usually considered to be more sensitive to OPs than explorative behavior such as our bungalow-test. On the other hand, the amount of stress inherent to restrainment was similar for all our marmosets, since they were first exposed to air and later to GB. The only difference was the type of exposure which was either air or GB. Although the present preliminary EEG data suggest that the EEG signal is more sensitive to GB than the eye, the EEG effects are more complex in nature than the more reliable miotic response. The effects on the EEG therefore require further investigation using more experimental animals. The more straightforward miotic response, showing a clear dose-relationship, might therefore be considered at this moment as the most reliable biomarker of exposure to low levels of GB.
Although the *LOAEL* value for the VER was as low as the *LOAEL* value for the EEG in guinea pigs, irrespective of the type pretreatment of these animals, *LOAEL* value for the VER in marmosets was much higher than that in guinea pigs (Table 30). It might be that miosis in marmosets interferes more with the light flashes to induce VERs than it does in guinea pigs. Also in this case our data are too preliminary to permit a firm conclusion.

Lack of dose-effect relationship on performance
In general, the *LOAEL* values for startle-response, shuttle-box and bungalow-test performances are much higher than those for miosis and EEG and therefore these parameters seem to be less relevant than the latter parameters.
In vehicle-pretreated guinea pigs GB-exposure in the dose-range tested had no effect on the startle-response (Fig 29), whereas shuttle-box behavior of the same animals revealed significant changes at all GB concentrations (except at 14.3 $\mu g/m^3$) (Fig 23). This observation suggests that for vehicle-pretreated guinea pigs the shuttle-box behavior is more sensitive for GB-exposure than the startle-response. In pyridostigmine-pretreated animals GB-exposure had only a significant effect on the startle-response at 50 $\mu g/m^3$ (Fig 30), whereas the shuttle-box behavior of the same animals revealed a significant effect only at 199.5 $\mu g/m^3$ (Fig 33). This lack of dose-effect relationship regarding these parameters should be attributed to the limited number of animals tested (one animal per GB concentration) and perhaps also to the limited dose-range tested. The latter remark also holds for vehicle- or pyridostigmine-marmosets regarding their startle-response (Fig 54 and 55, respectively) and their bungalow-test behavior (Fig 59 and 61, respectively). Transient significant changes were observed at only one of the various GB-concentrations the marmosets were exposed to. On the other hand these very preliminary data might indicate the threshold level range of GB exposure (7.5-150 $\mu g/m^3$) at which performance of guinea pigs and the marmosets start to become influenced.

Ad 3.
It is not clear from the present data whether unexpected adverse effects will emerge through the combination of pyridostigmine-pretreatment and exposure to GB. Nevertheless, the present preliminary data show that the *LOAEL* for EEG changes in both pyridostigmine-pretreated guinea pigs and marmosets was lower than for vehicle-pretreated animals. The *LOAEL* for the VER in marmosets, the *LOAEL* for the startle-responses of both species, and the *LOAEL* for the bungalow-test response of marmosets had lower scores in pyridostigmine-

pretreated than in vehicle-pretreated animals. In order to confirm such influence by pyridostigmine it should be further investigated.
It should be emphasized, however, that in both pyridostigmine-pretreated guinea pigs and marmosets, before the animals were exposed to GB, performance was significantly ($p < 0.05$) decreased compared to vehicle-pretreated animals, suggesting that pyridostigmine-pretreatment by itself decreased performance. For pyridostigmine-pretreated guinea pigs the shuttle-box behavior was significantly decreased (Fig 31), and for pyridostigmine-pretreated marmosets the bungalow-test response was significantly decreased (Fig 60 and 62) 1.5 h after a 5 h exposure to air only. It is not clear whether this phenomenon could be attributed to the combination pyridostigmine pretreatment and stress caused by restrainment. These findings may be relevant in view of the Gulf War illnesses since most veterans were pyridostigmine-pretreated and should therefore be confirmed by further research.

AChE-activity in blood
Exposure of guinea pigs to various concentrations of GB (in the range of 7.5 – 150 $\mu g/m^3$) in the present study, did not result in significant decreases in blood-AChE activities, as determined by the classic radiometric assay, neither in vehicle-pretreated, nor in pyridostigmine-pretreated animals (Figs 34 and 35). In contrast, the ChE activities in blood of marmosets were dose-dependently inhibited (Figs 63 and 64), being very pronounced at the higher dose-levels. This species difference might reflect the lesser amount of aspecific binding sites in blood of marmosets than in guinea pigs, which is also expressed in the more pronounced lethality in marmosets than in guinea pigs (Spruit et al 2000).
Using the fluoride-induced regeneration of GB from ChE in blood samples taken from animals at the end of the 5 h exposure to GB (in the range of 7.5 – 150 $\mu g/m^3$), it was found that in pyridostigmine-pretreated marmosets the GB concentrations were lower than that in vehicle-pretreated animals (not determined for guinea pigs). Presumably, this reflects the number of binding sites which is reduced (approximately 30%) through the presence of pyridostigmine, even at these low inhibition levels.

Occupational exposure limits and low level exposure
The estimated *LOEL* and *LOAEL* data should be addressed in the light of the recommended occupational exposure limits for GB vapor in air. In a recent publication on Airborne Exposure Limits for G-agents, the Research and Technology Directorate of Edgewood Research, Development & Engineering Center (ERDEC) recommended limits for occupational exposure, now referred to as "worker population limit (WPL), i.e., for workers without respiratory protection: a maximum averaged air concentration of 0.0001 mg/m^3 (averaged over an eight hour work day) (Mioduszewski et al 1998). This would correspond with a no-effect level (C.t-value) of exposure of 0.048 $mg.min.m^{-3}$. At this occupational limit even the mildest miosis or inhibition of AChE in blood should not occur.
The averaged *LOEL* value was 0.010 ± 0.002 and 0.04 ± 0.01 $mg.min.m^{-3}$ for vehicle-pretreated guinea pigs and marmosets, respectively. At these *LOEL* values, which are below the recommended no-effect level, fluoride-regenerated GB could be measured in blood samples. This means that the recommanded WPL should be reconsidered if not even the mildest ChE-inhibition in blood should occur.
The present study showed a significant ($p < 0.05$) decrease in pupil size (miosis) in vehicle-pretreated guinea pigs and marmosets during a 5 h (300 min) exposure to GB, corresponding to a *LOAEL* of 1.8 – 2.5 $mg.min.m^{-3}$. Assuming that this *LOAEL* is also valid for humans, miosis would be expected to appear by the end of a day (i.e. 480 min) at an exposure

concentration of about 5 $\mu g/m^3$ GB. The tolerable exposure concentration should then be << 5 $\mu g/m^3$ GB which is not in conflict with the above-mentioned recommendation of 0.0001 mg/m^3 GB.
In the above-mentioned publication by Mioduszewski et al (1998) the Short Term Exposure Limit (STEL) for GB is set at 2 $\mu g/m^3$ (15 min x 4/day). This would correspond with a no-effect dose level of 0.12 $mg.min.m^{-3}$ for four STEL exposures (60 min). In the present study GB could be regenerated from blood samples (ChE) within 60 min of guinea pig or marmoset exposure to GB concentrations in the range of 0.05-0.5 $\mu g/m^3$, corresponding with a range of C.t-values of 0.003-0.03 $mg.min.m^{-3}$, irrespective of the pretreatment. This suggests that the recommanded STEL level should also be reconsidered.
As mentioned before, a very preliminary finding is the higher sensitivity of the EEG for GB than the eye, which was observed in both guinea pigs and in marmosets (see Table 30), but this observation should further be investigated.

Field alarm and low level exposure
It is often suggested that it is difficult to develop detectors that are more sensitive than the miotic response of the human eye. Regarding the sensitivity of detectors in comparison with human thresholds for miosis, the Subcommittee on Toxicity Values for Selected Nerve and Vesicant Agents (NAS, 1997) estimates the miosis level for GB as 0.5 $mg.min.m^{-3}$ (or somewhat higher) based on ≤ 20 min exposures of humans to GB in the present study involving a 300 min exposure time, the *LOAEL* for GB regarding miosis, appeared to be in the range of 2.2-2.4 $mg.min.m^{-3}$ for vehicle-pretreated guinea pigs and marmosets. This corresponds with exposure to a concentration of GB of 7.3 – 8.0 $\mu g/m^3$. The M8A1 field alarm system, based on ion mobility spectrometry, which U.S. forces use widely, is designed to detect nerve agents as vapors or aerosols. It responds within less than 2 min to G-agents in the concentration range of 0.1 to 0.2 mg/m^3 (http://www.gulflink.osd.mil/library/randrep/bw_paper/, page 109). The U.K.'s CAM is a similar kind of detector, also based on ion mobility spectrometry, which responds to nerve agents at 0.1 mg/m^3 within less than a min. Evidently, the alarm will not detect significant miosis under these conditions of low level exposure.

Low level exposure on the battle-field
Various developments lead to the notion that the effects on military personnel of low level exposure to chemical warfare agents become increasingly important under actual battlefield conditions. Several realistic circumstances can be envisaged where low level exposure may take place: (1) Small amounts of agent may penetrate through the closures and through unnoticed slight damages to protecting clothing or gas masks. (2) Imperfections during donning and doffing procedures of protective gear will have the same effect. (3) Personnel performing duty in collectively protected areas may be exposed to small amounts of agent due to entry and exit procedures of the area and residual contamination of entering personnel. (4) Both offgassing and the physical contact with decontaminated material (painted surfaces, protective clothing) may contribute to low level exposure. (5) Possible exposure of personnel due to a downwind transport of an agent over long distances from contaminated areas, e.g. due to destruction of enemy stockpiles (suggested as a possible contributing factor to the Gulf War Syndrome; Ember 1996).
As mentioned above the alarm will not detect significant miosis or CNS neuronal transmission disturbances as reflected by presumed EEG deviations under conditions of low level exposure (present study).

As discussed above, it is not clear from the present data whether unexpected adverse effects will emerge through the combination of pyridostigmine-pretreatment and low level exposure to GB. Although the *LOAEL* for EEG changes in both pyridostigmine-pretreated guinea pigs and marmosets, the *LOAEL* for the VER in marmosets, the *LOAEL* for the startle-responses of both species, and the *LOAEL* for the bungalow-test response of marmosets had somewhat lower scores in pyridostigmine-pretreated than in vehicle-pretreated animals (Table 30), these very preliminary observations should further be confirmed.
As discussed earlier, pyridostigmine-pretreatment by itself interfered with performance before the animals were exposed to GB. It remains unclear, however, whether this phenomenon could be attributed to the combination pyridostigmine-pretreatment and stress caused by restrainment of the animals. Both may be relevant in view of the Gulf War illnesses.

Final remarks
Instead of adhering to certain definitions of "low level" exposure, the approach of the present study was to expose animals to the lowest controllable GB concentrations and to increase these concentrations untill GB could first be determined in blood samples *(LOEL)* and subsequently to levels at which adverse effects (miosis) became visible *(LOAEL).*
In order to achieve our goal, exposure equipment had to be developed and available analytical techniques for measuring very low levels of GB vapor in air (8-160 ppt) had to be tightened up which was very involved.
We ultimately succeeded in resolving these technical problems and were able to determine the *LOEL*, and the *LOAEL* for miosis in both guinea pigs and marmosets. These latter values have clearcut consequences for occupational and battlefield exposure to low levels of GB.
The remaining budget was then used to investigate other potentially interesting parameters such as EEG, VER, and performance in both species. The outcome of the latter investigations, however, should be considered as very preliminary and require further investigation for confirmation. This holds in particular for the observed EEG deviations at very low levels of exposure.
We recommand to use our now implemented and validated equipment (hardware and software) to determine the *LOEL* and *LOAEL* (miosis and EEG) of other nerve agents in a comparable way, especially of VX for which very insufficient data are available. An additional point of interest is the impact of wind speed on the development of miosis which might be nerve gas dependent.

KEY RESEARCH ACCOMPLISHMENTS

- The development of a validated system (hardware and software) to generate, analyse semi-continuously, and expose conscious animals (guinea pigs and marmosets) whole-body to low levels (lower limit: 8-80 ppt: 0.05-0.5 $\mu g/m^3$) of GB and probably to other nerve agents (soman, tabun, VX, cyclohexyl sarin, sulfur mustard) for several hours. During exposure, a number of parameters can be monitored on-line: miotic response by using digital cameras, EEG and VER telemetrically, blood samples can be drawn for internal dose assessment (fluoride-regenerated GB from blood ChE), AChE-activity in blood, and for toxicokinetic purposes (Annual Report 1998).
- The development of restraints for keeping conscious guinea pigs and marmosets for whole-body exposure over several hours (Annual Report 1998 and Final Report 2001).
- Implementation of software to analyse the miotic response on-line by determining the ratio between iris and pupil diameters (Annual Report 1998).
- Establishment of the Lowest Observable Effect Level (*LOEL*) for whole-body exposure of vehicle-pretreated and for pyridostigmine-pretreated guinea pigs to GB vapor in air (Annual Report 1999 and Final Report 2001).
- Establishment of the Lowest Observable Effect Level (*LOEL*) for whole-body exposure of vehicle-pretreated and for pyridostigmine-pretreated marmosets to GB vapor in air (Final Report 2001).
- Establishment of the Lowest Observable Adverse Effect Level (*LOAEL*) for miosis in guinea pigs. Moreover, preliminary *LOAEL* values were also estimated for EEG, VER, startle-response and shuttle-box behavior for whole-body GB-exposed guinea pigs which were either vehicle- or pyridostigmine-pretreated (Final Report 2001).
- Establishment of the Lowest Observable Adverse Effect Level (*LOAEL*) for miosis in marmosets. Moreover, preliminary *LOAEL* values were also estimated for EEG, VER, startle-response and bungalow-test behavior for whole-body GB-exposed vehicle-pretreated and for pyridostigmine-pretreated marmosets (Final Report 2001).
- Exposure of guinea pigs to low levels of GB vapor in the range of 7.5-150 $\mu g/m^3$ for 300 min did not result in significant ($p < 0.05$) decreases in AChE-activity in blood. In contrast, exposure of marmosets to similar GB concentrations for 300 min, resulted concentration-dependently in highly significant decreases of AChE-activity in blood.
- In pyridostigmine-pretreated marmosets exposed to GB vapor (in a range of 7.5-150 $\mu g/m^3$), the fluoride-regenerated GB concentration measured in blood samples taken at the end of a 5 h exposure, was lower than in vehicle-pretreated animals, presumably reflecting the lesser amount of binding sites in blood of pyridostigmine-pretreated marmosets and proof of enzyme-protection by pyridostigmine. This was not determined for guinea pigs.
- In guinea pigs and marmosets, before exposure to GB, performance was significantly ($p < 0.05$) decreased by pyridostigmine pretreatment. This finding may be relevant in view of the Gulf War illnesses in that most of the veterans were pyridostigmine-pretreated.
- Miosis and possibly EEG-changes may occur during long term (5 h) GB exposure at levels (far below 0.1 mg/m^3) which are not detectable by the currently available field alarm systems, assuming that humans are as sensitive as guinea pigs and marmosets for GB vapor in air.
- It is concluded that the recommanded occupational exposure limit (the Worker Population Limit, WPL) of 0.0001 mg/m^3 and the Short Term Exposure Limit (STEL) of 0.002 mg/m^3 for GB should be reconsidered if not even the mildest blood ChE-inhibition should occur, assuming that humans are as sensitive to GB as the experimental animals used.

REPORTABLE OUTCOMES

Van Helden HPM, Langenberg JP, Benschop HP (1998). Annual Report Grant Agreement DAMD17-97-1-7360.

Van Helden HPM, Langenberg JP, Benschop HP (1999). Annual Report Grant Agreement DAMD17-97-1-7360.

Van Helden HPM, Langenberg JP, Benschop HP (2000). Annual Reporting Grant Agreement DAMD17-97-1-7360 on the BioScience Rev.(oral presentation plus manuscript), Hunt Valley, Maryland, June 2000.

Van Helden HPM, Langenberg JP, Benschop HP (2001). Final Report Grant Agreement DAMD17-97-1-7360, March 2001.

CONCLUSIONS

- We have developed and implemented a validated system (hardware and software) for generating, analyzing semi-continuously, and exposing conscious animals (guinea pigs and marmosets) whole-body to low levels (lower limit: 8-80 ppt: 0.05-0.5 $\mu g/m^3$) of GB vapor and presumably to other nerve agents agents (soman, tabun, VX, cyclohexyl sarin, sulfur mustard vapor) for several hours. During exposure, a number of parameters can be monitored on-line: the concentration of GB in the exposure chamber, miotic response by using digital cameras, EEG and VER radiotelemetrically, whereas blood samples can be drawn for internal dose assessment (fluoride-regenerated GB from ChE in blood), AChE-activity in blood, and for toxicokinetic purposes. We recommend to use this system to determine the *LOEL* and *LOAEL* values of other CW-agents in a comparable way, especially of VX for which very insufficient data are available. The present finding that during a 5 h exposure of guinea pigs to 146 $\mu g/m^3$ GB there was no significant inhibition of blood AChE in contrast to earlier findings with a similar concentration of GD (Benschop et al 1998), prompt to investigate all nerve agents in this respect.
- An additional variable of this system is the wind speed in the animal exposure chamber caused by the air flow of 10 L/min, which may influence the impact of a nerve gas on miosis. Moreover, the influence of wind speed on miosis might be nerve gas dependent.
- It is highly relevant to investigate possible adverse effects in the above-mentioned low level exposure range, since field alarm will not go off at such low airborne levels of GB, whereas several potentially incapacitating effects (miosis, effects on EEG and VER, decrement in performance) may become significant as shown by the present study.
- The main results obtained with this system in the present study allow us to answer the major questions put forward at the end of the introductory paragraph:
  (i) What are the *Lowest Observable Effect Levels (LOEL)* for vehicle-pretreated and pyridostigmine-pretreated guinea pigs and marmosets? Established.
  (ii) What are the *Lowest Observable Adverse Effect Levels (LOAEL)* on performance (miosis, EEG, VER, startle-response, shuttle-box behavior, bungalow-test) for vehicle-pretreated and for pyridostigmine-pretreated guinea pigs and marmosets? Established for miosis, indicative for the other parameters mentioned.
  (iii) Do unexpected adverse effects emerge through the combination of pyridostigmine-pretreatment and GB exposure? Inconclusive answer.
- In both pyridostigmine-pretreated guinea pigs and marmosets, before exposure to GB, performance was significantly ($p < 0.05$) decreased compared to unexposed vehicle-pretreated animals indicating that pyridostigmine-pretreatment by itself decreased performance. This finding may be relevant in view of the Gulf War illness since many veterans were pyridostigmine-pretreated.
- Exposure of guinea pigs to low levels of GB vapor in the range of 7.5-150 $\mu g/m^3$ did not result in significant ($p < 0.05$) decreases in AChE-activities in blood, whereas exposure of marmosets to similar GB concentrations, resulted concentration-dependently in highly significant decreases of AChE-activities in blood.
- The finding that in pyridostigmine-pretreated marmosets exposed to GB vapor, the fluoride-regenerated GB concentration in blood was lower than in vehicle-pretreated animals, presumably reflects the lesser amount of binding sites for GB due to enzyme protection by pyridostigmine, even at these low inhibition levels.
- Miosis will occur during long term GB exposure to levels which are not detectable by the currently available field alarm systems, assuming that humans are as sensitive for GB vapor in air as guinea pigs and marmosets.

- The recommanded occupational exposure limit (the Worker Population Limit, WPL) of 0.0001 mg/m^3 and the Short Term Exposure Limit (STEL) of 0.002 mg/m^3 for GB should be reconsidered if not even the mildest effects (blood ChE-inhibition and EEG effects) should occur.

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U.S. ARMY CHEMICAL AND BIOLOGICAL DEFENSE COMMAND

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EVALUATION OF AIRBORNE EXPOSURE LIMITS FOR G-AGENTS: OCCUPATIONAL AND GENERAL POPULATION EXPOSURE CRITERIA

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13. ABSTRACT *(Maximum 200 words)*

Existing occupational airborne exposure limits (now referred to as "worker population limits" or WPLs) and general population airborne exposure limits (now referred to as "general population limits" or GPLs) were reviewed and recalculated using current risk assessment methods and incorporation of data not considered in previous estimates. The existing WPLs for agents GB (0.0001 mg/m^3), GA (0.0001 mg/m^3) and GD (0.00003 mg/m^3) were deemed adequately protective. Similarly, GPL values were recalculated and found to be adequately protective for GB (0.000003 mg/m^3) and GA (0.000003 mg/m^3). A GPL value of (0.000001 mg/m^3) was recommended for GD. New WPL (0.00003 mg/m^3), and GPL (0.000001 mg/m^3) exposure guidelines were developed for GF, for which none previously existed. The existing acute exposure guidelines for occupational workers, namely those for Immediately Dangerous to Life or Health (IDLH), were recalculated resulting in the following limits for a 30 min exposure: GA (0.1 mg/m^3), GB (0.1 mg/m^3), GD (0.05 mg/m^3), and GF (0.05 mg/m^3). Short-Term Exposure Limits (STELs), limited to 15 min for up to 4 times in an 8 hr work day, were developed for occupational workers for GA (0.002 mg/m^3), GB (0.002 mg/m^3), GD (0.001 mg/m^3) and GF (0.001 mg/m^3). Likewise, Acute Exposure Guideline Levels (AEGL-1) were developed for the general population for exposure durations of: a) 30 min for GA (0.0024 mg/m^3), GB (0.0024 mg/m^3), GD (0.0012 mg/m^3) and GF (0.0012 mg/m^3) b) 1 hr for GA (0.0012 mg/m^3), GB (0.0012 mg/m^3), GD (0.0006 mg/m^3) and GF (0.0006 mg/m^3) and c) 4 hrs for GA (0.0003 mg/m^3), GB (0.0003 mg/m^3), GD (0.0001 mg/m^3) and GF (0.0001 mg/m^3).

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Airborne Exposure Limits (AELs); Soman (GD); Tabun (GA); Sarin (GB); Vapor; Inhalation; Human exposure; GF

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EXECUTIVE SUMMARY

PURPOSE

The purpose of this document is threefold:

(1) The adequacy of existing G-agent (GA, GB, GD) airborne exposure limits (AELs) for the occupational setting and general population are evaluated on the basis of currently accepted risk assessment approaches as well as through the incorporation of any relevant data which has become available since the time the existing AELs were first derived.

(2) AELs are also derived for the nerve agent GF, for which there are no existing criteria.

(3) Currently accepted risk assessment methodologies are also used to derive additional exposure criteria which did not previously exist. Specifically, short-term exposure limits (STELs) for the occupational setting as well as acute exposure guideline level one (AEGL-1) for the general population are derived.

DISCUSSION

The G-type chemical warfare (CW) agents include Sarin (GB), Tabun (GA), Soman (GD) and GF, which are organophosphate ester derivatives of phosphoric acid. Small quantities of CW agents or agent by-products are used by various military and contract laboratories for defensive research purposes, and verification of Chemical Weapons Convention compliance. Although bulk quantities are no longer manufactured in the United States, they currently exist in military stockpiles where they await eventual destruction.

People whose work environment may include chemical weapon materials, whether in storage depots and demilitarization facilities, laboratory research, verification of the Chemical Weapons Convention, remediation and decontamination, or emergency response operations, face potential risks of accidental exposure to these materials. This risk is also shared to a much lesser extent by the general population in communities surrounding areas where chemical agents are stored, transported or processed for disposal. In addition, chemical weapons, whether in foreign or domestic stockpiles, are still considered potential military threats and terrorist targets. The most likely route of exposure is by inhalation, but also may include the direct effects of chemical agent vapor on the eyes.

Existing AELs for GA and GB were promulgated by the CDC (DHHS, 1988); DA PAM 40-8, and DA PAM 385-61 also provide AELs for GD. These AELs include 8 hr/day; 5 day/week TWA, and IDLH (30 min) guidelines for the occupational setting as well as a 72 hr TWA for the general population. However, it should be noted that the latter guideline (general population AEL) is, in fact, a 24 hr/day; 7 day/week TWA for an estimated lifetime exposure. The original AEL was expressed as a 72 hr TWA only to reflect sampling requirements at the time of the original CDC publication (DHHS, 1988).

The process used to derive the existing AELs did not necessarily conform to today's accepted methodologies. In addition, certain additional data and studies have become available since the time of their derivation. The use of additional data and methodologies presumably will allow greater certainty in estimating concentration guidelines which are protective of occupational personnel and the general population.

FINDINGS AND CONCLUSIONS

Findings and conlusions resulting from recalculation of existing exposure criteria and development of new crtiteria include the following:

(1) The recalculation of existing occupational AELs resulted in concentration values with 2-3 fold differences. In terms of uncertainties inherent in the risk assessment process, these values are deemed within an acceptable range of each other. Therefore, the existing occupational AELs are deemed valid and adequately protective. Recalculated general population AEL values were also similar to existing criteria values. In order to differentiate between the long-term and short-term AELs, occupational worker AELs are referred to as worker population Limits (WPLs) and general population AELs are referred to as general population limits (GPLs).

Note:

(a) The recommended AELs are estimates associated with "no observable adverse effects" in (i) the workforce for an 8 hr/day TWA; 40 hr week, for a lifetime, and (ii) in the general population for a 24 hr/day; 7 days/week, for a lifetime.

(b) Unlike the above "no observable adverse effects" for AELs, the biological endpoint selected for determining the IDLH estimate includes generalized weakness, and signs of systemic G-agent poisoning in addition to less serious effects including miosis, rhinorrhea, and tightness of the chest. IDLH estimates are limited to acute exposures (up to 30 min).

(2) The estimated STELs and AEGL-1 concentration values are presented in the Table below.

Note:

(a) Exposures above the TLV-TWA up to the STEL should be no longer than 15 min, and should not occur more than four times per day. The developed STEL values are based upon acute human exposure data and estimate airborne concentrations associated with "no observable adverse effects" in humans (chemical workforce population).

Recommended Airborne Exposure Limits (AELs) for GB, GA, GD, and GF in Occupational (WPL) and General Populations (GPL)

Recommended AEL (mg/m^3)				
GB	GA	GD	GF	Application
Occupational Worker AELs (WPLs)				
0.0001	0.0001	0.00003	0.00003*	WPL (TWA; 8 hr/day, 5 days/wk)
0.002*	0.002*	0.001*	0.001*	STEL (TWA; 15 min x 4/day)
0.1	0.1	0.05	0.05*	IDLH (30 min)
General Population AELs (GPLs)				
0.000003	0.000003	0.000001	0.000001*	GPL (TWA; 24 hr/day 7 days/wk)
0.0024*	0.0024*	0.0012*	0.0012*	AEGL-1 (30 min)
0.0012*	0.0012*	0.0006*	0.0006*	AEGL-1 (1 hr)
0.0003*	0.0003*	0.0001*	0.0001*	AEGL-1 (4 hr)

* = Developed (no existing criteria).
WPL = Worker population airborne exposure limit or Occupational AEL (no observable adverse effects)
GPL = General population airborne exposure limit or General population AEL (no observable adverse effects)
IDLH- = Immediately Dangerous to Life or Health
STEL = Short Term Exposure Limit
AEGL-1 = Acute Exposure Guideline Level -1
TWA = Time Weighted Average

(b) The acute exposure guideline levels limited to discomfort (AEGL-level 1) are estimates for acute (30 min, 1 hr, and 4 hr) exposure scenarios associated with the lowest observable adverse effects (miosis, rhinorrhea and tightness of chest) in humans (general population).

(3) The AELs for agent GF are presented in the Table below. These values will be necessary where GF is identified or potentially present.